

Priceless proposals

Recommendations for a reorganization of US research on the environment are vitiated by the absence of any indication of the likely costs.

ENVIRONMENTAL research in the United States is carried out by a number of government and private organizations that are not in any serious way coordinated with each other. The demands for such research in the 1990s, and for channelling its findings to the makers of policy, are of a different order from those of the 1960s and 1970s that spawned the current system. Then, worry about energy and pollution was especially high in the public consciousness; now it is, for instance, climate change and groundwater contamination, along with a catalogue of other insults to the natural world too depressing to recite. So there is a crying need for a restructuring of US government support for work on environmental issues, not least to take account of their increasingly global aspects.

These are the premises of a task force set up by the Carnegie Commission on Science, Technology, and Government, an independent advisory body, which after two years' gestation has now given birth to its report *Environmental Research and Development: Strengthening the Federal Infrastructure*. An ambitious document it is too.

The report leaves no stone unturned. Most notable among the recommendations are that an Institute for Environmental Assessment should be established to provide the analysis necessary for policy formulation; that the 12 national laboratories of the Environmental Protection Agency (EPA) should be consolidated into four, with the creation of some six other research institutes in universities and elsewhere; and the merger of the US Geological Survey with the National Oceanic and Atmospheric Administration to create a US Environmental Monitoring Agency. In essence, this last is an old chestnut, having been first mooted during the Nixon years.

Here, then, the Carnegie Commission has tackled some pressing matters of high significance. But, sad to say, it has ducked the two big questions.

The first is what research needs to be done, and what can now be dispensed with (or, put brutally, is second rate). The explicit concern of the report is process and organization, but that is a crippling restrictive mandate. The commission points, for instance, to the widely acknowledged shortcomings of the EPA's programmes but charitably puts them down to inadequate organization and funding. The agency's incoming administrator, Carol Browner, will have to be harder-headed on that score and place quality of science at the top of the EPA's agenda.

The second is the question of cost — both of whether the

estimated \$5 billion spent on environmental research and development in 1992 is enough for the tasks to be undertaken, and of how much the restructuring would itself consume. What realism there is on that score is of the vaguest kind. "Many of the recommendations ... can be implemented without significant new federal expenditures ...". Really? And with "large [budget] deficits expected in future years, the federal government will be constrained in its ability to address environmental needs". There is the rub.

As President-elect Bill Clinton puts together his new administration, the United States has paused for contemplation of the future, economic affairs being at the centre of the navel-gazing. This well-intentioned and otherwise sensible document will find its way into many a waste bin before the year is out. That is a pity, but will be a consequence of the commission's failure to put a price tag on its proposals. Other groups are now labouring on similar reports that will have to plunge into the treacherous waters of identifying what science is to be done, and what the cost will be, if they are to carry any clout. □

Norplant for teenagers

The city of Baltimore will offer Norplant in health clinics affiliated with public schools.

THE incidence of teenage pregnancy in the United States (and in other nations as well) can be described as a public health crisis. The woman whom US President-elect Bill Clinton has designated as the next Surgeon General says that even in Arkansas (a midwestern state of 2,372,000) some 8,000 teenage girls have unwanted pregnancies in any given year. Stemming this epidemic of children having children has been difficult for a number of reasons, among them the fact that many teenagers fail to use birth control pills, condoms and other available contraceptive methods consistently.

Norplant, Baltimore city health officials decided, may be the answer — at least for girls who are willing to choose long-term contraception. Pellets surgically injected under the skin will release contraceptive hormones for five years.

Although the cost of Norplant, at anywhere from \$350 to \$500, may seem high, it is actually less than a five-year supply of the pill and is a wise investment of public funds as long as teenagers are not coerced into accepting it. Other school systems should follow Baltimore's example. □



Survey finds that restructuring has improved UK university research

London. The wave of closures and mergers of university science departments in Britain over the past few years has significantly increased the quality of the research being produced, according to figures released last week by the Universities Funding Council (UFC). The figures come from an assessment of the research output of every university department in the country, based primarily on a detailed study of published articles.

Some 62 panels of expert reviewers looked at the work of 43,000 academic staff from 172 institutions. Under the terms of the exercise — conducted to help the newly created Higher Education Funding Councils for England, Wales and Scotland decide how to allocate £650 million (US\$1 billion) next year for research — each department was rated on a scale of one (low) to five (high). Three years ago, the average score for all university departments across the country was 3.2; this year it has risen to 3.7.

Critics say that the figures are misleading and that the higher score may reflect merely an improved ability to impress a panel of outside reviewers. Although each researcher was allowed to present only two scientific papers, concern remains that the quantity of research results may still outweigh quality or that a relatively brief contribution to a well-known scientific journal may have greater impact than a more substantial contribution to a less prominent journal.

But those responsible for the assessment believe that the exercise provides clear evidence of an improvement in research output. This change, they argue, has been the result of decisions by some universities to close down departments that scored badly on previous exercises or to merge them with stronger departments.

They give as an example the high marks scored by a group of university Earth-science departments, each of which has been a focus for government support following the recommendations of the Roxburgh report in the mid-1980s. Similarly, all the university chemistry departments receiving a score of one in the 1989 exercise have since been closed or merged, boosting the overall ratings of the universities concerned.

As in the previous exercise, the Universities of Cambridge and Oxford emerged with the highest ratings. Some 40 of 52 departments at Cambridge were awarded the maximum of five points, representing work of international excellence. Oxford lagged behind, scoring top marks in 28 of 45 departments.

Other institutions that scored highly in the overall ratings included University

College, London, with 22 departments receiving highest marks; Imperial College, London, with 12 departments; and the Universities of Warwick and Nottingham, with 11. The University of Edinburgh, with 11 top-rated departments, headed the list of Scottish universities.

Several universities — in particular, Bath, Keele, Brunel and Lancaster — showed a marked improvement in their overall scores. This reflects efforts to weed out weak spots and build up strengths. At the same time, the review panels awarded only one point to 20 “old” university departments; as a result, these are unlikely to receive any funds for research next year.

This year’s exercise was the first time that the research in the so-called “new universities” — previously polytechnics or similar colleges that have recently been allowed to call themselves universities — had been assessed. Although many of these scored low marks on the ratings, 12 departments

received a score of four, indicating that the work meets high national standards.

“The existence of pockets of high-quality research shows that many of these ‘new’ universities have done well, particularly with only limited funding to develop their research potential”, says Graeme Davies of the UFC.

The Association of University Teachers, which represents academic staff in pay negotiations with the government, called the exercise an “impressive achievement” given declining government support for research. But the union warned that the results may have been influenced by the methodology used — in particular, by the freedom given to university departments to decide how many of their research staff to include in the exercise. “It is possible for two departments with identical strengths to have taken different decisions about including staff and to have been given a different — and thus flawed — rating”, it said. **David Dickson**

Reports examine US universities

Washington. Two reports issued this week (21 December) on the health of US research universities* serve as reminders of how easy it is to propose what should be done and how hard it is to bring about substantive change.

At what may be his final press conference, D. Allan Bromley, whose tenure as assistant to the president for science and technology ends next month, expressed his pleasure at presenting the recommendations of both the President’s Council of Advisors on Science and Technology (PCAST) and the Federal Coordinating Council for Science, Engineering and Technology (FCCSET) on ways in which the nation’s 150 to 200 chief research universities could maintain US leadership on the frontiers of science. That feeling is not surprising, given that in 1986 he and industrialist David Packard, as members of PCAST’s predecessor, the White House Science Council, produced a report containing some of the same recommendations, including full reimbursement for the cost of federally funded research and an end to congressional earmarking of research funds awarded without scientific review.

The 46-page PCAST report makes a point of saying that universities must weed out those research programmes not of world-class quality because the US government can no longer afford to support an ever-expanding academic research enterprise. And it tells government agencies not to create

programmes “that would increase the net capacity of the system”. Yet it calls for a “substantial” federal programme to repair and renovate ageing academic research facilities and a new federal programme of undergraduate scholarships. The facilities programme, long opposed by the Bush administration, could easily cost hundreds of millions of dollars annually and the scholarships, if successful, would eventually increase the number of academic scientists and, thus, the demands for funding from the federal government.

Harold Shapiro, president of Princeton University and vice chairman of PCAST, said that the report asks research universities “to put quality ahead of quantity and scope”, reversing a trend since the 1960s of aspiring to greatness in an increasing number of fields. Such a policy also flies in the face of a programme begun by the National Science Foundation and now spreading to half a dozen research agencies that helps have-not states to do better in national competitions for federal dollars. “I don’t think it’s wise to increase the base when you can’t adequately support what already exists”, said Shapiro, who added that universities must decide for themselves what departments to trim or eliminate.

Jeffrey Mervis

* The PCAST report is *Renewing The Promise: Research-Intensive Universities and the Nation*; the FCCSET report is *In the National Interest: The Federal Government and Research-Intensive Universities*.

University of California forms company to develop technology from its laboratories

San Francisco. Seed money from the University of California (UC) would fund start-up companies based on faculty inventions under a plan to bolster projects languishing between government funding and commercial development. A new, for-profit Technology Development Corporation, of which the university would own 51 per cent, would also pay to develop ideas from campus and

one-third of the total would go into university coffers, with the rest earmarked for inventors and to pay fees and lawyers.

UC has more than 1,000 inventions looking for companies, said Carl Wootten, director of the UC Systemwide Office of Technology Transfer. He estimated that only about half are far enough along to interest industry.

Some projects would continue in on-campus

laboratories or move to the federally funded Los Alamos, Livermore and Lawrence Berkeley Laboratories. A sterling few would merit startup financing from the development company, in tandem with standard venture capitalists willing to share the risk. The university would own part of the companies and share royalties with its venture-capital partners.

The new company would help to make university inventions more attractive as well as generate revenue for the UC system, Wootten said. He

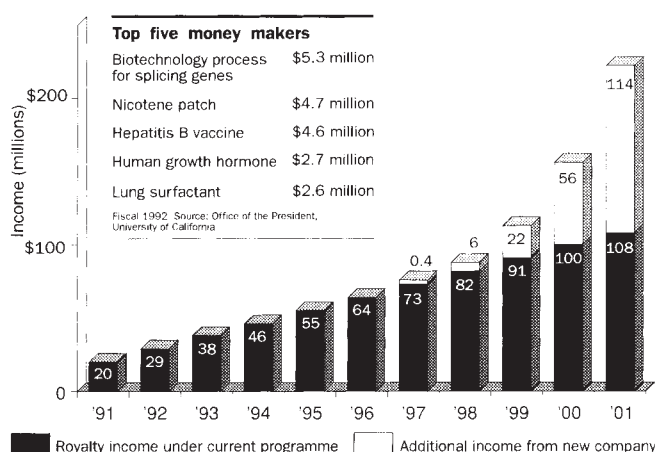
The University of Chicago has started nine companies to develop faculty inventions through its own \$9-million seed venture capital fund. The fund is restricted to university ventures but still provides investors with the chance to invest early in promising new companies, said Robert Nelsen, manager of its not-for-profit parent.

Johns Hopkins has operated a for-profit arm, called Triad Investors Corporation, that provides "bridge" funding for inventions. The company is not limited to supporting university inventions.

Massachusetts Institute of Technology, the leader in university patenting and licensing with about two licences issued a week, owns stock in many licensees, but limits its involvement to 20 per cent or less. It does not finance start-up companies for fear of appearing to be profiting from government-funded research.

Sally Lehman

UC pins its hopes on patents



Figures estimated for 1993–2001.

government laboratories into prototypes that outside companies could license.

The university hopes to raise \$100 million from established companies for the development of technology contributed by the university. UC also hopes to persuade the state to turn over its share of royalty income to help fund the venture, which it expects will cost \$2 million a year to operate. Next year, it hopes to spend \$2.5 million on development projects on campus or in government laboratories and \$500,000 on two startup companies.

The university helped to give birth to the \$6-billion biotechnology industry through the original technique for splicing genes, for which it shares a patent with Stanford University. Its inventions generate more royalty income than any other US university, some \$29 million in fiscal year 1992 alone (see above).

Jack W. Peltason, UC president, outlined the programme before the university's governing body on 10 December, saying that the goal is to harness faculty brain power to stimulate economic growth and create jobs for California. He estimated that it could generate \$222 million in gross royalty income by the year 2001. The money would provide a much-needed boost to the cash-strapped UC system, although only about

believes the university could have reaped another \$50 million from previous inventions if it had asked for equity in the start-up licensees.

University officials will try to persuade the state to turn over its share of royalty income — 25 percent after expenses and payments to inventors — to pay for operations. Additional money would come from selling stock to long-term investors such as major corporations and retirement systems.

The development company would not fund projects invented outside UC, university officials said, even though outside investors would be participating. And, because the university holds the patent to all campus inventions, faculty members would have to bring their inventions first to the UC technology transfer office.

Industry observers are withholding judgement on the proposal, which the university hopes to implement in 1993. Gary Hooper, vice president of business development at Cygnus Therapeutic Systems in Redwood City, California, expressed concern about potential restrictions from outside investors and emphasized the importance of scientists setting their own research agendas.

Similar on-campus funding mechanisms have grown more popular in recent years.

Germany get price break from CERN

Munich. Germany has reached an agreement with CERN (the European Laboratory for Particle Physics) to reduce its contribution by SFr25 million (US\$18 million) for three years because of the high cost of reunification.

At its December meeting, CERN announced that it would give Germany a rebate of 10 per cent on past contributions in recognition of "special circumstances", a reference to its having allowed other former communist countries to join CERN on the cheap. CERN officials wanted to avoid offering Germany a straightforward discount on fees, contrary to its rules, for fear of other countries asking for a similar reprieve.

Half of the shortfall in CERN's annual budget of SFr950 million will be made up by holding increases in salary and material costs to 2.4 per cent instead of the 3.6 per cent that would match inflation. The rest will be made up by general economy measures in the laboratory. There will be no staff redundancies.

Hermann Strub, head of the German delegation to CERN, said that taking on the 18 million citizens of the former East Germany raised Germany's contribution — determined by a complex algorithm that depends primarily on gross national product — close to the limit of 25 per cent of its budget that CERN can receive from one country. At the same time, Strub emphasized Germany's full commitment to CERN, in particular the Large Hadron Collider (LHC) planned for later in the decade.

Alison Abbott

German state unexpectedly approves first gene trials

Heidelberg, Germany. A state ethics panel has given its approval for the first gene therapy trials in Germany — and the first gene therapy directed against cancer in Europe. The decision upsets the conventional wisdom that it is futile even to apply for permission because of the prevailing hostility in Germany towards any form of genetic engineering.

Gene laws in Germany are much more stringent than in any other country. Some state authorities have been accused of deliberately overinterpreting the letter of the law to delay licensing for as long as possible because of political opposition to genetic engineering. As a result, most German scientists working in the field look for collaborators in other countries.

But a proposal from Roland Mertelsmann of Freiburg University Medical Centre to treat cancer patients by stimulating their immune system was approved within three months without a hitch. "I faced a challenge", he says. "Everyone told me that it would be impossible, but in fact it was very straightforward."

Data in Germany must be registered with the Public Health Office and the Paul Ehrlich Institute, which controls use of vaccines. Formal approval is given by an ethics committee within each state, in Mertelsmann's case, Baden-Württemberg.

Anticipating resistance, Mertelsmann chose a transfection method that does not use a retrovirus. His alternative — electroporation — is a standard method not often used because of its low efficiency.

To compensate, Mertelsmann developed a method that uses an abundant tissue. The gene for the cytokine interleukin 2 (IL-2) will be inserted into autologous fibroblasts cultured from skin biopsy of patients with either renal cell carcinoma, colon cancer or malignant melanoma. (These cancers were chosen because they occur frequently, are well-characterized and are known to be responsive to cytokines). Once stably transfected, a process that could take as long as nine months, the fibroblasts will be mixed with cancer cells from the patient, irradiated and injected subcutaneously as a vaccine.

The rationale behind this novel approach is to activate *in vivo* a specific population of cytotoxic T cells, which will then attack tumour cells. Cytotoxic T cells require two signals to be activated. The first recognizes specific cancer-associated antigens on the tumour cells themselves; they can then be triggered by an appropriate cytokine such as IL-2. The vaccine provides both signals to T cells circulating in the patient's blood that are known to infiltrate the injection site. It is hoped that this approach, when optimized,

will pick off metastatic cancer cells after surgical removal of the primary tumour before they have a chance to become established.

Mertelsmann's methodology has some theoretical advantages. In the United States, Steven Rosenberg of the National Institutes of Health has been criticized for using genetically modified tumour infiltrating lymphocytes (TILs) because the transformation of the cells somehow interferes with the ability of the TILs to home in on the tumour (see *Nature* **360**, 399, 1992). By using a vaccine, Mertelsmann hopes to invoke a normal *in vivo* immunological response against the tumour or its metastases.

Mertelsmann makes no claim for therapy. Indeed, the time required to develop the vaccine means that metastases have the opportunity to develop beyond a stage where such therapy could be expected to work. Although his procedure is at this point experimental, he says that it will not harm the patient and should provide important information about whether T cells specific for the tumour cells are activated by this approach *in vivo* and whether they are functional.

Data on animal toxicity will be submitted shortly to the Paul Ehrlich Institute, which makes recommendations about safety for vaccines in Germany. Mertelsmann



Mertelsmann gets straight answer.

hopes to enrol his first patients by April.

The approval has caught German colleagues by surprise. Delores Schindel, of the Institute of Immunology in Munich, is also working on treating the same cancers with IL-2 transfected lymphocytes, but decided not to request permission to conduct trials in Germany. Instead, she establishes her cell lines in Munich and accompanies them to the United States, where she collaborates with the Sloan Kettering Institute for Cancer Research in New York on retroviral transfection experiments. She then flies the engineered cells back to Munich, where she uses certified laboratories to prove that no active retrovirus remain. The cells can then be used experimentally.

Although the procedure is cumbersome, she believes that crossing the Atlantic is easier than trying to initiate clinical work in Germany. In the meantime, she says, there is much to be done on animals.

Alison Abbott

Japan delays SSC decision, awaits Clinton

Tokyo & Washington. Japan has managed yet again to postpone a decision on contributing to the construction costs of the \$8.5-billion US Superconducting Super Collider (SSC) project, this time citing the need for US President-elect Bill Clinton to make clear his intentions.

It is nearly three years since the United States started putting pressure on Japan to contribute significantly — to the tune of about \$1.5 billion — towards the proton-proton collider being built in Texas. At the end of 1991, just before a visit to Japan by the US president, George Bush, there were even signs that the Japanese prime minister, Kiichi Miyazawa, was going to create funds for the SSC in a new budget item for "international collaboration".

But politicians and industrial leaders refused to support the extra taxes that would be needed. Instead, the two leaders agreed last January to form a US-Japan working group that would reach a decision on Japan's participation by the end of the year.

Last week, however, an official of Japan's Science and Technology Agency confirmed that "both governments have agreed

to postpone a final decision" because of the imminent arrival of the Clinton administration. This effectively means that Japan will not spend any money on the SSC in its next fiscal year, which begins in April 1993, unless Miyazawa takes the unusual step of creating a supplementary budget.

US officials play down the delay and insist it will not affect the future of the project. But William Happer, director of the Office of Energy Research in the US Department of Energy, says that time is running out for the SSC project to incorporate any form of in-kind contributions. "If it's cash, we can get it later without much problem", he says, "but [a donation of] equipment would stress our capacity to handle it smoothly".

The House of Representatives of the US Congress has told organizers to get one-third of their money — roughly \$2.8 billion — from non-federal sources. Apart from the state of Texas, which is providing \$1 billion in support, Japan is the only possible source of a large contribution. Other potential contributors, such as Taiwan, are expected to take their lead from Japan.

David Swinbanks & Jeffrey Mervis

Germany follows Britain in dumping fast breeder

Munich. Europe's fast-breeder reactor programme is on the verge of collapse because Germany has joined Britain in pulling out of its commitment. France is now the only country with a continuing interest in the programme, and it may not be able to carry on alone.

On 8 December, Germany announced that funding for fast breeder research at the Karlsruhe Nuclear Research Centre will not be renewed after 1993. Nuclear power is controversial in Germany, which has shown a strong commitment to the environment, and no issue is more sensitive than the use of plutonium in fast-breeder reactors. Thus it was no surprise that when the British government decided to abandon the 1984 trilateral agreement to develop a European fast-breeder reactor (see *Nature* **360**, 93; 1992), Germany reversed its own position, expressing its "respect for the decision" and saying that it was a "consequence of an energy policy appropriate to the times".

The decision is a great disappointment to researchers, who say that a research programme that took years to build up has been dismantled overnight. "From 1994 onwards, the future looks very sad", says Willy Marth, executive director of the management group for the European fastbreeder programme. "Everything we've worked for has just been brushed away."

The anticipated cost of the project was an important factor for both countries, trying desperately to trim spending. French officials acknowledge that the outlook for fast breeders, which looked bright when the joint venture was proposed eight years ago, is much dimmer now. But Philippe Hammer, deputy director of nuclear reactors at the French Atomic Energy Commission, says that the commission hopes to obtain enough additional money from its other sources of funding, the government and the utilities company EDF, to be able to go it alone, although at a slower pace. He believes that fast breeders are an inevitable part of the long-term future of energy

generation in Europe.

The loss of British and German support also threatens reprocessing facilities in France and Britain. German's decision caused dismay at Britain's Sellafield plant, with reports that two German companies, RWE and Pilz, were planning to renege on their current reprocessing contracts.

The rumours were fuelled by continuing debates in the German parliament about the atomic energy law. The law, passed in the 1970s, requires all spent fuel to be reprocessed; at the time, it was believed to be the most efficient way to dispose of radioactive fuel. But times have changed and the law is expected to be modified, probably in 1994, to allow industry to choose between reprocessing and storage.

The utilities companies have been quick to contradict reports that they want to pull out immediately. "Whatever happens, we will be fulfilling our contracts with THORP at Sellafield and with the French reprocessing plant Cogema", says an RWE spokesman, who explained that the rumour stems from a misreading of a letter from the industry to Chancellor Helmut Kohl, agreeing simply to take part in talks on the future of nuclear power in Germany. The contract is unlikely to be renewed upon expiring in 2000, however, as storage has become cheaper than reprocessing.

The long-term feasibility of THORP is threatened by this development, particularly because its second line of activity may also be doomed. Reprocessing plants are pinning their hopes on the sale of reprocessed plutonium in fuel rods called MOX elements, which can be burnt in conventional light-water reactors. But even this market is shrinking as nuclear power falls out of favour. Britain has no plans to build any more light-water reactors (it has only one, Sizewell B) and Germany looks increasingly likely to make a political decision against using the plutonium-based fuel in its reactors.

Alison Abbott

EC expected to rearrange research portfolio

Munich. The European Commission was expected this week to agree to divide its research programme between two commissions. Meeting in Strasbourg, the Council of Ministers is prepared to shift industrial research, along with high-profile innovation and technology transfer programmes such as SPRINT, into a separate commission to be headed by Germany's former minister of economic affairs, Martin Bangemann. Filippo Pandolfi has for the last few years

held responsibility for both industrial and fundamental research.

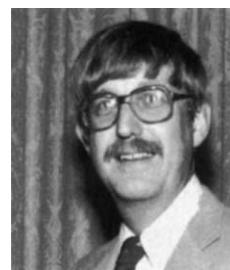
Antonio Ruberti, a former minister of research in Italy, is favoured to assume the job of commissioner of the remaining section supervising basic research. It was rumoured at one point that education would be combined with the non-industrial research portfolio, but it is now thought likely to remain a separate section.

Alison Abbott

Collins to head NIH genome centre, open laboratory

Washington. Officials at the US National Institutes of Health (NIH) have succeeded in recruiting Francis Collins, a geneticist at the University of Michigan, to become director of NIH's National Center for Human Genome Research. Collins, who expects to move to the NIH campus in mid-1993, will also form an intramural research programme at the centre, which until now has only funded genome research around the country.

Collins has long been the leading candidate to succeed James Watson, who resigned last spring after questions were raised about his holdings in various biotechnology companies (see *Nature* **356**, 549; 1992). Much of the speculation



Francis Collins

has centred on whether NIH would be able to make its offer attractive enough to lure Collins away from Michigan, where he has worked since 1984. The recruitment campaign appeared to suffer a setback in October, when Congress cut by nearly 50 per cent the \$20-million discretionary fund that Bernadine Healy, the NIH director, had planned to draw on to provide a new laboratory for Collins. But Collins, whose Michigan laboratory costs approximately \$1.5 million a year to run, says that the discretionary fund will be big enough to support his new laboratory.

NIH is expected to request additional money for the genome centre in its budget request for fiscal year 1994, which begins on 1 October 1993. Collins's appointment, although a virtual certainty, must be approved by the director of the Department of Health and Human Services, NIH's parent organization.

Collins is expected to bring with him much of his research team, which numbers 25. A co-discoverer in 1989 of the gene for cystic fibrosis, Collins will probably occupy laboratory space vacated by geneticist Craig Venter, who left NIH last July to direct a new, private company to do large-scale gene sequencing (see *Nature* **358**, 95; 1992). Collins also plans by 1995 to add 20 principal investigators and their research teams to the centre to work on gene discovery, a field largely neglected on the NIH campus, and on gene therapy.

Traci Watson

Academic freedom in Greece

SIR — I wish to report a development in Greece that represents a flagrant violation of academic freedom. The conservative government has passed a law with barely any debate essentially abolishing the university reform act of 1982. This law has been passed despite the overwhelming protests of university rectors, faculty and students. It is offered as a "modernization" of the higher education system, but in effect it takes us back to the Middle Ages.

About 18 months ago, the education minister asked the university community for its views on the then-existing law (reform act of 1982). Although the questionnaire was criticized, because in many of the multiple choice questions all available choices, except "other", were variations of those that the minister himself favoured, the minister later admitted that the majority of respondents did not want any changes.

The 1982 act allowed collective government of the universities by administrators, faculty and students, but the minister of education has opted for a scheme where most decisions are made by one person (rector, chairman of a department).

The 1982 act allowed for all faculty positions to be potentially tenure-track, with the two lower positions (lecturer and assistant professor) nontenured and the two higher (associate professor and professor) tenured, thus allowing the younger generation of scholars to do research and to be assessed on the basis of their performance. The result has been a tremendous increase in scholarly work, as a cursory look in the *Science Citation Index* will show, since the junior faculty were no longer under the immediate authority of the professor. With the new law a freeze is put on all promotions, while positions at the junior level are no longer tenure track, but temporary. In a country with very little employment in the private sector for scientists with PhDs, this is tantamount to scaring younger scientists away from a university career. Tenure positions under the new law will be announced every four years, and the exact allocation per discipline, department and school is to be decided by the minister himself. So instead of worrying about doing their academic jobs well, the younger faculty must vie with each other to become favourites.

As if that were not enough, the new law mandates part-time faculty as well as "full-time but not exclusive" faculty, a contradiction in terms. While the 1982 reform act established full-time faculties, it unfortunately did not prohibit outside employment, which for those in the

professions can be very lucrative. Indeed, the new law seems to attack those who take to heart the full-time principle of university work, and strive day after day for the betterment of Greek higher education. For many of the older professors, their job is a ticket to the social circuit, and many of them are barely conversant with their subject.

Last but not least, under the new law, students will now have to pay for their books, except those who are poor or whose parents are tax-dodgers. This will certainly force book prices sky high, not to mention the danger of reverting to the days when the purchase of the professor's expensive but worthless text at a specified bookshop was often the gateway to passing his course.

I can only venture that this new law is intended to result somehow in "private universities" springing up all over the land. This idea was half-tried last year, when the previous minister of education quietly allowed several unscrupulous individuals to open such "universities", charging enormous fees and being nothing more than rented buildings with a few rooms, with many of the old professors lending their names to such monstrosities for "prestige". While the government was ridiculed over this disastrous attempt at privatization, the new law seems simply to be a more refined approach in the hopes of returning to the good old days.

Many universities have now been taken over by the students in protest, and the faculty associations are boycotting examinations. No one knows what will become of the universities, but the minister and the 13 wise old professors behind this new law can take pride in having put back Greek higher education a few decades. After all, the minister himself has yet to venture to a European Communities education ministers' meeting after 18 months on the job, so can we expect him to know any better?

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Polish pollution

SIR — Recent correspondents have drawn attention to air pollution as a possible cause of lung cancer and other diseases¹⁻³. But many Western epidemiologists have never been confronted with the degree of air, water and soil pollution affecting Eastern European countries. I live in Upper Silesia, southern Poland, a heavily populated

region where air pollution has reached unprecedented levels. In Upper Silesia, there is continued exposure to high and constant concentrations of suspended particulates, PAHs, SO₂, NO₂, phenol, formaldehyde, metals and so on, and the level of exposure to, for example, benzo(a)pyrene in ambient air exceeds by a factor of 3-5 the level of a passive smoker⁴. Cluster-type mortality from lung and larynx cancer (as also cancer at other sites) was noted in Silesia by the epidemiological section at our institute (B. Zemla, unpublished observations). In the most polluted part of Silesia, the standardized mortality index from lung cancer in men amounts to 117 deaths per 100,000 (compared with the Polish 'average' of 69). This illustrates the usefulness of 'mapping' in small areas of cancer incidence or mortality in the search for aetiological factors compared with using 'average' numbers for a wider region, and raises the question of what is responsible for such geographical differences.

"Project Silesia", supported by the Environmental Protection Agency (EPA), recently reported high cancer risk due to coke oven emissions in Ostrava and Frydek-Mistek (the Czechoslovak part of Silesia). There are only scant data indicating a possible synergistic interaction between cigarette smoking and air pollution. Interactions between various causative factors (cigarette smoking, radiation, former infections, environmental or work-place pollutants) deserve further study.

Roughly one child in six in central Silesia suffers from a chronic upper respiratory tract disease. What are the consequences? Rearrangements of chromosomes, a high rate of sister chromatid exchange, mitotic arrest and a high level of DNA adducts were found in a pilot study of Silesian inhabitants as compared to rural control group⁵⁻⁷. Are these findings meaningless for human health?

To begin to understand the complex basis of lung cancer, we need to know not only how much people smoke, but also the quality of air that people have been breathing all their lives, as well as information about familial risk factors.

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800 actors in search of a strategy

David Dickson

The British government's request for input into its promised White Paper on science has stimulated an unprecedented and often controversial stream of suggestions from the scientific community.

Not since the Rothschild report of more than twenty years ago has Britain's scientific community experienced such ferment over plans for its own future. At that time, it was Lord Rothschild's proposal that applied research be placed on a "customer-contractor" basis that stimulated heated exchanges from the floor of the House of Commons to the letters columns of *The Times*.

Today, with the Rothschild principle now firmly established in government thinking, the new threat is the proposed "privatization" of government-funded research. The trigger to the current ferment has been last July's request by William Waldegrave, the Chancellor of the Duchy of Lancaster, for suggestions on what should be included in a White Paper on the organization of science that he has promised for early next year. About 800 proposals have been received in reply, and are now being scrutinized by officials in the Office of Science and Technology (OST), which reports to Waldegrave in his position as gov-

ernment minister responsible for science.

Of these, the biggest number (31 per cent) have come from higher education institutions. Slightly fewer (29 per cent) have been received from companies, business organizations and professional bodies; 12 per cent from other organizations inside and outside government (including charities and trade unions); and the remaining 28 per cent from members of the public or "otherwise unassigned."

Last Friday, the OST announced that copies of the replies from over 200 organizations were being placed in the Library of the House of Commons and the British Library. A sampling of these submissions shows that many repeat familiar complaints — such as the underfunding of the research base, or the lack of job security for research workers on short-term contracts. "Those parts of the science base on which industry depends are now in a perilous state", says the submission from the chemicals firm ICI. "Any further decline in some

sectors may be terminal."

At the core of the submissions, however, is a clear divergence of views on a central question that will have a profound effect on the future of many thousands of British scientists: how far can (and should) free market principles be applied to the organization, funding and execution of scientific research? In particular, can government-funded science be privatized in the same way as, for example, electricity production or the collection of statistics?

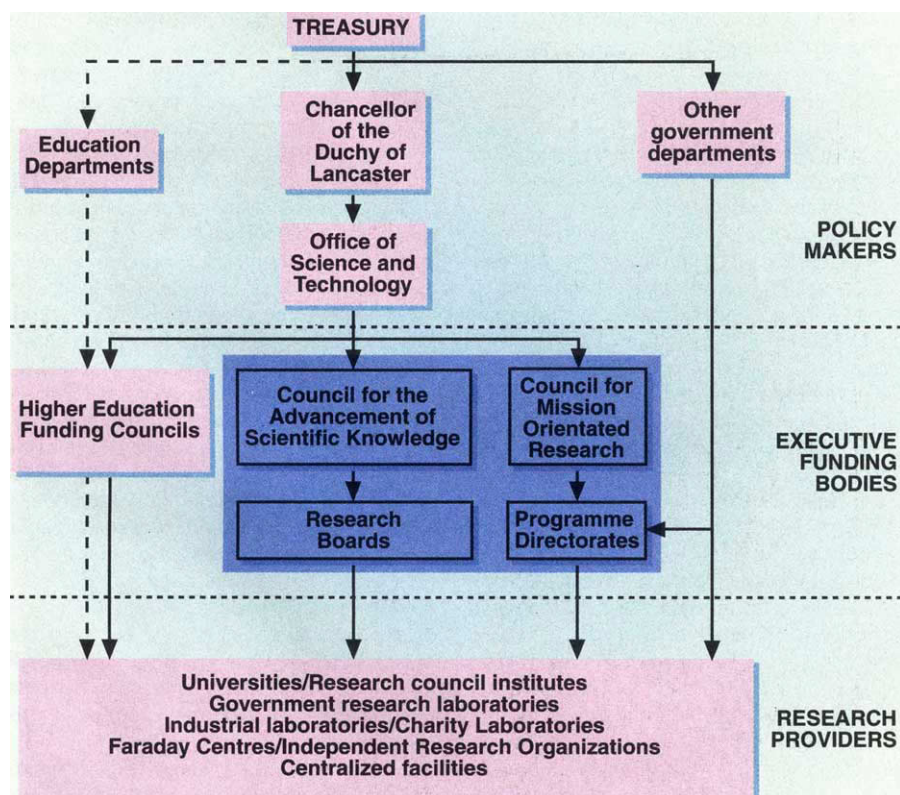
A number of relatively uncontroversial themes run through many of the submissions, and it would be a surprise if they did not figure prominently in the final White Paper. One is the desire for a strong statement from the government of the value that it places on the work of the scientific community in the context of its desire to stimulate industrial innovation — a statement that could be used as a framework for debates on how the two can best interact.

Linked to this is support for the idea that the government needs an explicit strategy for science and technology. The consensus on this point is broad. "There needs to be a consistent pattern in the attitudes of government departments towards the support that is given to innovative ideas," says the Defence Manufacturers Association. The union Manufacturing, Science, Finance (MSF) asks for "a government strategy for R&D and innovation which ensures a coordinated and consistent policy across the private and public sectors."

In the light of the creation of the new OST, it is not surprising that one broadly backed proposal is for a reshuffling of the government's top advisory boards. The general thrust here is the government needs a single, all-powerful committee reporting on all aspects of science and technology policy directly to the Cabinet. In practical terms, the most obvious step, as suggested in several submissions, would be to upgrade the role of the current Advisory Council on Science and Technology (ACOST).

One important reason for this is to broaden the base of decisions about science. "A [single] advisory board, which must have a wide-ranging membership from outside government, and indeed from outside the membership of the research councils themselves, is essential if the country is not to run the risk of scientific policy becoming dangerously inbred", says the University of Cambridge in a submission from its vice-chancellor, David Williams.

There also appears to be a general



The shape of science funding to come? The Advisory Council on Science and Technology has recommended a new system for financing research, with a strict division into three levels of organization, and a new set of bodies (in the shaded area) replacing the current research councils.

consensus on the need to pay greater attention to the European dimension of science policy. Many submissions support the view of the Royal Society, either explicitly or implicitly, that it is essential to incorporate an awareness of the research activities of the Commission of the European Communities in Brussels into the baseline of British science and technology policy.

Replies were invited by Waldegrave to the question: Do we get optimum benefit from our international collaborative programmes, especially in the European community? It is clear, from the virtual unanimity of the replies received, that Britain does not. The Defence Manufacturers Association answers straightforwardly: "This is a key question, and the answer is NO."

The government also needs to take note of the breadth of support for the idea that it should use the White Paper as an opportunity to address the question of subscriptions to international scientific organizations. Paying these, as at present, out of the annual allocations of the research councils means that domestic scientific programmes frequently fall victim to fluctuations in the international currency markets. There is a growing consensus that some protection is needed. ACOST, like many other bodies, recommends that funding for participation in major international organizations and facilities that are the subject of intergovernmental agreements "should be the direct responsibility of OST".

Not surprisingly, many organizations have used the opportunity of Waldegrave's invitation to put forward suggestions that would directly improve the situation of their own members. The Institute of Electrical Engineers repeats the arguments for a separate Engineering Research Council, staffed by "engineers with a strong industrial background", on the grounds that engineering remains a junior partner within the Science and Engineering Research Council. This move is strongly supported by the Royal Academy of Engineering — but equally strongly resisted by the SERC itself, which argues that a split between science and engineering would be both artificial and damaging.

A plea comes from the Institute of Physics for the government to recognize the economic importance of "highly competitive and dynamic physics-based industry sector", and urges "sufficient funding for both physics teaching and research" to produce the trained individuals required in all sector of the economy. Representing the view of about 2,000 expatriate scientists, British Scientists Abroad calls on the government to "initiate an active policy to make it possible for top expatriate scientists to return to Britain," and makes a special request for the research councils to institute six-year, two-phase grants for postdoctoral and mid-career study overseas which provide "assurance of funding on return to the UK".

Nor have the unions which represent

scientific staff, such as the Institute of Professionals, Managers and Specialists, lost the opportunity to argue their case for greater security of employment for scientists. "The growth of fixed term contracts should be halted and reversed," says the IPMS, arguing that "such contracts have become a major barrier to the recruitment and retention of high quality staff". The staff of the Medical Research Council go further to suggest that the government should set up an inquiry into the career structure of scientists.

If there is an element of special pleading in each of these suggestions, it is also to be found in one of the main themes where opinions begin to diverge significantly, namely on how the government should balance its support to basic and applied research. At the one end of the spectrum, organizations whose principal concern is the healthy operation of the science base argue that this needs protection if it is to remain effective. At the other end are submissions from industrial bodies who make it clear that they are unhappy with the amount of support that basic science receives.

BT (British Telecom) suggests explicitly that "resources should continue to be directed away from 'big science' towards technologies associated with generating products and services." "The undirected use of public resources to support science and technology [is] a luxury we cannot afford," says The Centre for the Exploitation of Science and Technology (CEST).

There is no shortage of proposals for alternative schemes to the current arrangement of five research councils. The ABRC, for example, argues that the scientific responsibilities for the present Royal Society proposes a new research council to cover aspects of basic research, particularly in respect of the core physical science, together with a small number of mission-oriented councils, each covering both basic and strategic research relevant to their missions.

At the other end of the spectrum, the most radical proposal comes from CEST, which suggests that all public spending on research — including basic science — becomes the responsibility of a new, private Industrial Research Council. "Industry, the 'customer' for research and development services, has to be empowered to manage the public funds deployed and evaluate the performance of 'suppliers'", says CEST.

In between the two ends of the spectrum lie ACOST's proposal, which has already attracted wide attention, that publicly-funded research should be channelled through two main bodies: a Committee for the Advancement of Scientific Knowledge (CASK), which would finance curiosity-driven research through a number of research boards; and a Council for Mission Orientated Research (CMOR), operating through a number of programme directorates.

The philosophy behind the ACOST proposals, written in a language and from a point of view that have led many to treat it as

a possible first draft of the government's final position, is that a clear separation is needed between the way in which the two types of research are handled.

Not surprisingly, this proposal has already become the focus of a considerable amount of criticism. Last week, for example, the Committee of Vice Chancellors and Principals (CVCP) followed up its earlier submission to OST with a further statement in which it disagreed strongly with this idea. "It is not possible to draw a clear distinction in such terms from the point of view of either purchaser or provider," says the CVCP, words that are being widely echoed elsewhere in the scientific community.

The most controversial part of ACOST's proposal results from a similar desire to make a clear distinction between three different types of organization, occupying different levels in the funding hierarchy. At the first level would be policy-making bodies (such as OST) that prepare advice, policies, priorities and criteria for funding science. The second level would be made up by executive funding bodies responsible for providing funds to researchers according to these criteria and priorities. The third level would include the "research providers", ranging from university science departments and research council institutes to industrial laboratories.

Few dispute the functional principles on which this separation is made. Where there is disagreement, however, is on the nature of the links that should exist between the three levels. In particular, should the government merely lengthen the umbilical cord connecting the research councils to their institutes; or should the cord be cut completely?

ACOST argues for complete separation. "Research council laboratories and research institutes and department research establishments should become freestanding bodies competing with other research providers on equal terms," it says. What it describes as "the market for the supply of research services" would be strengthened by allowing all research providers equal access to sources of funds for research.

But ACOST's suggestion that the solution lies in an open-market approach remains open to question. Many feel that, in its enthusiasm to introduce more flexibility into both the funding and conduct of research, the council may be proposing throwing out the baby with the bathwater. A commitment to the long-term remains an essential element of support for science. Few trust that the market alone — whatever protection is built into future contractual arrangements between purchaser and providers — can provide this commitment.

In this context, the views of the Department of Trade and Industry will prove vital to the final shape of the White Paper. But open government still has its limits; those views will (at least officially) remain under wraps until the White Paper is published. □

When climate and life finally devolve

It is traditional, as the year draws to a close, to try to predict what the future holds in store. It is less common to ask, as Caldeira and Kasting do in this issue, "how much future?"

NEW calculations by Caldeira and Kasting on page 721 of this issue¹ are about the destiny and ultimate demise of the biosphere — which is both dependent on and vulnerable to the evolution of our main-sequence star, the Sun. Their calculations provide the best estimate to date as to when the Earth's staple of abiotic and biotic processes that draw down atmospheric CO₂ will be unable to offset a rising solar flux.

Predictions of the heat death of the biosphere have traditionally come from astronomers. But ten years ago, Earth scientists Lovelock and Whitfield showed that the biota would face a crisis² long before our planet is scorched as the Sun expands into a red-giant phase, in about 5 billion years. The crisis will be a scarcity of atmospheric CO₂.

The trend over geological time has been for atmospheric levels of CO₂ to decrease. (Current rising levels of CO₂ from industrial and agricultural sources are, by comparison, just a blip on the geological landscape.) Driving this long-term steady decrease has been a combination of factors³: the growth of continents, a declining geothermal heat flux, a sequence of evolutionary developments that increase the weatherability of the terrestrial surface, and an increasingly radiant Sun, all of which alter the dynamics of the geochemical system that balances the CO₂ supplied by volcanism with its removal by global weathering. The ultimate consequences of this downward trend were spelled out by Lovelock and Whitfield: in just 100 million years, CO₂ will drop below the minimum level necessary to support photosynthesis.

Caldeira and Kasting have recalculated the life span of the biosphere, using a more refined set of biological and physical inputs. These include a precise greenhouse function, increased photosynthetic efficiencies from C4 plants at reduced CO₂ and allowance for a functional dependence of silicate weathering upon respiration from roots and soil organisms. With these modifications, Caldeira and Kasting have extended the tenure of the biosphere to ten times that predicted by Lovelock and Whitfield: we can breathe easy for about a billion more years. But finally, temperatures will soar to the upper limits tolerable to the most primitive microbes, and in another billion years again the Earth will undergo a final sterilization with the

loss of the hydrogen from photolysed water to space.

Is an event so distant in time any cause for concern today? We certainly have more immediate problems during the coming century in trying to prevent our own selves from ravaging the biosphere. Nevertheless, Caldeira and Kasting's work raises issues that are hauntingly familiar: the role of CO₂ as a greenhouse gas, a possible need for planetary engineering in maintaining the Earth's habitability (or rather its comfort for humans), and fundamental questions about the ultimate persistence of life and the pace of evolution.

None of the models, of course, allows for possible evolutionary innovations that might buy time for life as we know it (or do not know it). The current plenitude of life's biochemical cycling mechanisms — within cells, organisms and ecosystems — is impressive, and has probably evolved in response to times of scarcity⁴. For example, C4 plants (such as maize) concentrate CO₂ in special bundle sheath cells, away from the sites of wasteful photorespiration; this innovation has evolved in the past 100 million years⁵, probably in response to falling levels of atmospheric CO₂.

Future responses to scarcity could be technologically driven. In experiments for advanced life support, such as NASA's programme in closed ecological life-support systems, Biosphere 2 in Arizona, and the Russian Bios 3, carbon is assiduously cycled and CO₂ is managed at levels well above that of the Earth. Such research is intended to give us the rudiments of understanding that could someday lead to the propagation of mini-biospheres across the Galaxy. The future ancestral Earth — dry, burnt, and dead — would then be fondly recalled in Heinleinian space songs as that mythic place of "cool green hills".

Our descendants or descendent species would not have to run from the devolution, however — they could fight. Shades in space or mirrors on the Earth that keep out a small fraction of the elevated future solar flux would be an option. Carbonates could be heated to release CO₂, a technique proposed⁶ for the industrial stewardship of the carbon cycle on a 'terraformed' Mars⁷ to make up for the lack of the CO₂-generating plate tectonics that we have on Earth.

More importantly, long before the

solar-induced devolution of the biosphere comes to pass, the ability of *Homo sapiens* to persist will be tested not only by our own earthly transgressions, but by impacts from asteroids and comets. An impact that could destroy civilization and wipe out a quarter of the world's population is estimated to occur about every 500,000 years⁸.

Calculations of the life span of the biosphere bring to focus one additional matter of great philosophical import. If devolution-computing life forms evolved with just a billion years to spare, following a 3.5 billion year genealogy, we are presented with an intriguingly close temporal match between two essentially decoupled, developing systems. The time it takes for life and then technological intelligence to evolve is nearly identical, in the only case we know, to the window of opportunity afforded by an atmospheric resource, which is yoked, in part, to the luminosity history of a main-sequence star. That this window was open just long enough for our arrival will add impetus to the arguments used by proponents of the anthropic cosmological principle⁹, who find the Universe uncannily fine-tuned.

Overall, Caldeira and Kasting's study adds to our understanding of what Lovelock calls geophysiology and NASA calls Earth-system science: the Earth as an interconnected system of life, atmosphere, hydrosphere and lithosphere, a system whose organizational properties we are still discovering. While our concerns over habitability are often for the shorter term, in exploring the interplay of life and climate over geological timescales, we forge an appreciation for where knowledge must go. **Tyler Volk**

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Mice with half a mind

Nigel Holder and Malcolm Maden

THE effect of the putative morphogen retinoic acid on the developing vertebrate nervous system has been brought into sharper focus by the paper by Marshall *et al.* on page 737 of this issue¹. Using a line of transgenic mice in which a reporter gene is linked to the promoter for one of the *Hox* genes implicated in brain development, they show clearly that retinoic acid treatment at the gastrula stage leads to a transformation in the rhombomeres of the anterior hindbrain region in which rhombomeres 2 and 3 are respecified as 4 and 5 respectively.

Retinoic acid has attracted much attention over the past few years because of the compound's remarkable effects on the tissue pattern of developing limbs and the discovery of the burgeoning family of nuclear retinoid receptors. Less attention has been paid to the effects of retinoic acid on the developing vertebrate nervous system². Most notable of these effects is the truncation of anterior head and brain structures and abnormal formation of the hindbrain, highlighted in 1989 by Durston *et al.*³, after treatment of *Xenopus* gastrulae with retinoic acid.

Durston and colleagues suggested that the abnormal phenotype was caused by respecification of anterior brain structures — forebrain and possibly anterior midbrain — into more posterior hindbrain. This neatly unified the action of retinoic acid on the patterning process within the embryo, as it also posteriorizes limb cells. But Marshall *et al.*'s results confirm work carried out by various groups over the past three years which has pinpointed the anterior hindbrain as the site of the retinoic acid effect. The respecification of anterior hindbrain is the common and predictable effect of exposure of gastrulae of various vertebrates — mouse, chick, *Xenopus* and zebrafish — to a short burst of retinoic acid⁴⁻⁷.

This effect on the hindbrain tied the teratology to another action of retinoic acid: its regulation of *Hox* gene complexes in a time- and dose-specific manner^{8,9}. These are the very genes that show limits of anterior expression patterns among the segmental units of the hindbrain¹⁰. This link prompted several groups to examine the expression pattern of relevant *Hox* genes in the anterior hindbrain of embryos treated with retinoic acid. *Hox-2.9*, which is normally expressed in the hindbrain only in rhombomere 4, is overexpressed more anteriorly^{4,11}.

Here the story has rested up until

now. The abnormal expression of *Hox-2.9* and other segmentally restricted genes such as *Krox-20*^{4,6,11} provided a link to the altered morphology but could not be clearly related to the acquisition of segmental identity for each of the rhombomeres. Marshall *et al.* carried out the same basic experiment as before: embryos *in utero* were exposed to retinoic acid at the gastrula stage, but two new features of their experimental design have revealed these striking new results. First, they used a line of transgenic mice carrying a *lacZ* reporter gene under the control of the *Hox-2.9* promoter to visualize the expression of the *Hox-2.9* gene in experimental animals. Second, the embryos were examined at stages later than in previous studies.

At the early stages of hindbrain formation in these embryos, *Hox-2.9* expression shows the predicted anterior expansion; however, in embryos allowed to develop a little longer this homogeneous expanded anterior region resolves into two clear stripes of *Hox-2.9*. What was an ill-defined anterior hindbrain seems to produce two rhombomere 4s separated by a clear rhombomere width. Probing with *Krox-20*, which normally expresses in rhombomeres 3 and 5, strongly suggests that this rhombomere is a second rhombomere 5, giving a pattern of a 4-5-4-5 repeat in the anterior region followed by the normal posterior rhombomere pattern. Anatomical analysis using backfills of fluorescent dye to label identifiable motor nuclei confirms the interpretation derived from the molecular markers.

The cells expressing *Hox-2.9* abnormally in the anterior hindbrain must interact in some way to cause the formation of a clear anatomical unit. Whatever the mechanism proves to be, it is likely to tell us something direct about the normal formation of the hindbrain rhombomere pattern and the role that *Hox* genes play in establishing segmental identity. The other crucial finding from these studies is that downstream retinoic acid effects lead to a clear respecification of anterior rhombomeres to posterior rhombomeres. The patterns generated in the current experiments appear to replace rhombomeres 2 and 3 with 4 and 5. No additional segmental units are described, although it is unclear what is going on with rhombomere 1 and the hindbrain-midbrain border region. Marshall *et al.* also show that respecification of rhombomeres leads to respecification of neural crest derivatives associated with each segment. The retinoic acid

therefore alters the anatomy of the head as a consequence of altered behaviour of respecified crest cells, reinforcing the view that the *Hox* code is crucial for the patterning of the head as a whole, not simply for the central nervous system.

A major question remains about these and related experiments. What is the primary effect of retinoic acid at the gastrula stage? The identification of this effect will be instructive because it may reveal the normal chain of command of interactions leading to the control of *Hox-2.9* expression in rhombomere 4 and to the expression of other regulatory genes in the hindbrain. It is important to know, for example, if the retinoic acid effect on *Hox-2.9* is direct. In the mouse, this gene and its paralogue in the *Hox-1* cluster, *Hox-1.6*, are expressed during gastrulation, as are their homologues in other species¹²⁻¹⁴. Interestingly, the *Hox-1.6* gene has recently been shown to contain a retinoic acid β receptor response element in the 3' region¹⁵, although it is not yet known whether this is functional. It may be the case, then, that the primary retinoic acid effect is on expression of the *Hox-1.6* gene, which then secondarily regulates *Hox-2.9* expression. Consistent with this notion is the intriguing observation that the phenotype of the *Hox-1.6* mutant mouse produced by homologous recombination^{16,17}, is grossly similar to the phenotype generated by retinoic acid in vertebrate embryos. Both generate clear-cut alterations to the development of the hindbrain region of the head. □

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Multi-talented stem cells?

Mike Dexter and Terry Allen

WHEN Lady Macbeth said "yet who would have thought the old man to have had so much blood in him", she had no notion of how much younger than the victim that blood was. The granulocytes in the blood had been released from his bone marrow only a few hours earlier, and the red cells within the previous few weeks. The reason, of course, is that the vast majority of blood cells are destined to die within a short time of being made and so must be continuously replenished from a pool of more primitive cells resident in the bone marrow. These are known as stem cells, and their quantitation, functional potential and purification have been major goals for haematologists for many years.

The prevailing view now is that stem cells represent perhaps 0.01 per cent of bone marrow cells, that they can self-renew, and that they can be assayed by their ability to regenerate the bone marrow and to give rise to long-term (life-time) lympho- and myelopoiesis¹. Erythrocytes, neutrophils, eosinophils, basophils and mast cells, monocytes and tissue macrophages, including their more specialized relations, such as osteoclasts, dendritic cells, Langerhans cells and Kupfer cells, and T and B lymphocytes, can all be produced from a single haematopoietic stem cell. A formidable task! But not so, it would appear, for the stem cell. Now a letter by Huang and Terstappen on page 745 of this issue² presents data that may extend this potency even further.

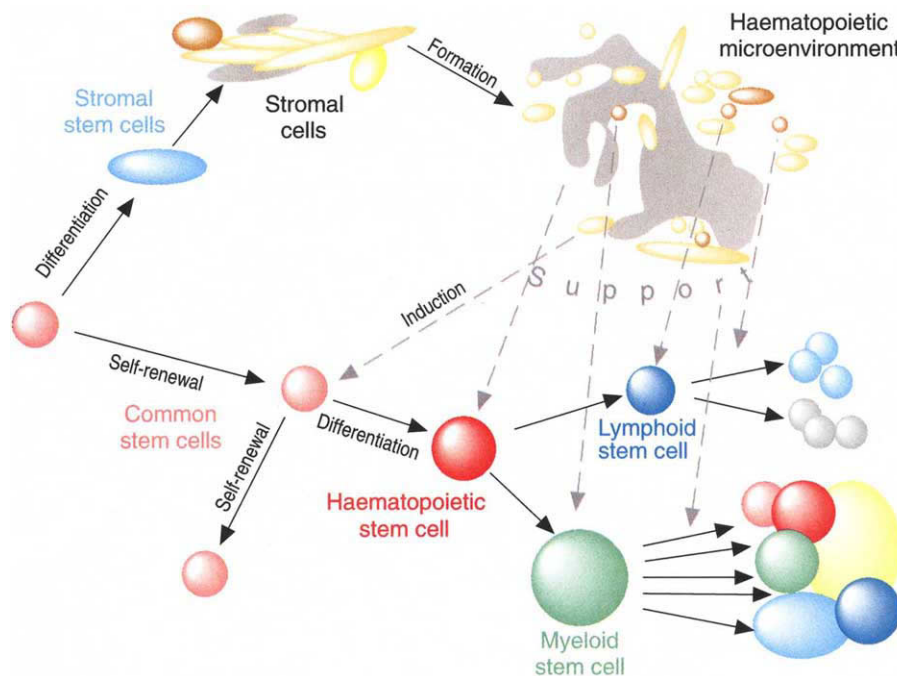
For some time it has been known that the putative haematopoietic stem cells in human marrow express the cell-surface antigen CD34 (ref. 3). Using a fluorescence-activated cell sorter and three-colour fluorescence, Huang and Terstappen have sorted these CD34⁺ cells further on the basis of their expression (or lack of expression) of HLA-DR and CD38. Those CD34⁺ cells that express either HLA and/or CD38 demonstrate typical haematopoietic cell developmental options. Only 6 per cent of the CD34⁺HLA-DR⁻CD38⁻ fraction could form haematopoietic cell colonies when supplied with the appropriate growth factors. This 0.01-per-cent fraction of the initial marrow cell harvest is exactly the estimated 'correct' value for the stem cell population. What is much more intriguing, however, is that when apparently single cells from this cell fraction were cultured in medium containing only the insulin-like growth factor IGF-1 and basic fibroblast growth factor as additional growth factors, between 1 and 12 per cent of the wells pro-

duced cultures of stromal cells with associated haematopoiesis; that is, 'single' cells can apparently reconstitute the haematopoietic stromal cell microenvironment of the bone marrow in addition to lymphoid cells and myeloid cells.

As the stromal cells are themselves heterogeneous (containing fibroblasts, adipocytes, smooth muscle cells, endothelial cells and osteoblasts), this is a

Even here, the well established phenomenon of emperipoiesis (viable cells enclosed within the cytoplasm of another cell)⁵ — also observed by us with time-lapse video in long-term marrow cultures — needs to be considered. Consequently, are the primitive haematopoietic cells simply being carried intracytoplasmically as a 'passenger' of the stromal stem cell?

Furthermore, if haematopoietic cells and bone marrow stroma share a common stem cell, why has no evidence emerged for such a cell from bone marrow transplantation? Although it was claimed some years ago that the bone



Pathways of differentiation of haematopoietic stem cells. (After Huang and Terstappen.)

quite staggering result: it increases the developmental options of the (bone marrow) stem cell considerably and also raises some serious questions regarding the target cells and pathogenesis of haematopoietic diseases.

How definitive, then, are the data that lead the authors to draw this conclusion? One worry is that the CD34⁺HLA-DR⁻CD38⁻ fraction of cells immediately after sorting contains contaminating "primitive mesenchymal elements". Is it possible that some of these cells become physically associated with the primitive haematopoietic cells and that these doublets are then sorted as 'single' cells? The authors claim not, on the basis of the frequency of 'doublets' (about 0.1 per cent of the wells) that are found after sorting single fluorescent beads measuring 6 µm. But beads are not cells — and the more pedantic of us would perhaps be more satisfied if direct visual examination of the wells had confirmed the statement or, preferably, following micromanipulation of single cells in addition to visual evaluation⁴.

marrow stroma was transplantable^{6,7}, subsequent studies⁸ did not support this idea. But if there is indeed a common stromal/haematopoietic stem cell, and if this cell contributes to haemopoiesis after transplantation, perhaps evidence for this would have been more forthcoming? Furthermore, bone marrow cells from patients with clonal haemopathies such as chronic myeloid leukaemia, acute leukaemias, myeloproliferative and myelodysplastic diseases have been extensively investigated and yet, to our knowledge, these studies without

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exception show that the bone marrow stromal elements are not part of the malignant clone^{9,10}. Such data do not, of course, rule out the possibility that the mutation(s) giving rise to the disease occurs in a more developmentally restricted cell and not in the common haematopoietic/stromal stem cell, but it is nonetheless surprising that no examples are found in which both cell populations are involved. It is possible, of course, that the common stem cell is restricted to the fetus, and is lost during acquisition of an adult phenotype.

Although the results of Huang and Terstappen are highly attractive in that they lend weight to a logical extrapolation of a haematopoietic tissue developmental hierarchy, we must treat them as an intriguing observation that will require further evidence for total confirmation. □

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ARCHAEO GEOLOGY

Vestiges of a beginning?

Stuart Ross Taylor

TANTALIZING geochemical evidence is presented by Harper and Jacobsen, on page 728 of this issue¹, that the Earth's first crust formed nearly 4,500 million years ago. Whereas the Moon's origin and evolution are understood both in principle and in detail, a veil falls across the Earth's history at around 4,000 million years ago. It is in the oldest rocks, of about this age, that geologists seek hints of the events of the preceding several hundred million years; but, as a debate developing around the new results attests, certainty is elusive at this juncture.

There is a general, if not universal, consensus that the Earth and the other terrestrial planets were formed by accretion, in a gas-free environment, from a hierarchy of planetesimals that included objects exceeding the size of Mars. This process² probably took somewhere between 10 and 50 million years after the formation of the solar nebula, 4,560±5 million years ago (Myr). Early melting of the Earth is an inevitable consequence of this planetesimal hypothesis, according to most workers. The incoming planetesimals, by analogy with the asteroids, were probably already chemically differentiated and possibly molten³. And the formation of the Moon seems inescapably linked to the collision with the Earth of an object of at least 0.15 Earth-masses (an impact of this magnitude is required to produce the angular momentum of the Earth-Moon system). Such a massive event would have melted the Earth, regardless of its previous accretional history⁴.

Given an initially molten Earth, what happened next? The Earth's metallic core must have settled out while the accretion still continued, but current geochemical opinion is divided over the subsequent crystallization history of the terrestrial mantle. From familiar examples of the crystallization of large bodies

of molten rock (such as the Skaergaard and Stillwater intrusions), one might expect the production of zones of differing mineralogy whose isotopic and elemental fingerprints would remain to inform us of this early event. Moreover, there is well-documented geochemical evidence that the Moon underwent something similar. Starting with a 'magma ocean', certainly involving half and possibly the whole Moon, crystallization of this silicate mass resulted in the flotation of a feldspathic crust (10–12 per cent of lunar volume). This is dated at 4,440 ± 20 Myr. The interior from which the mare basalts (the familiar dark patches that form the Man in the Moon) were erupted later, was solid by 4,400 Myr with the last dregs, strongly enriched in elements such as the rare-earth elements, crystallizing by 4,360 Myr.

Some of these circumstances were unique to the Moon. Feldspar was buoyant in the bone-dry lunar melt, but sinks in wet terrestrial magmas. Relative to the Earth, the Moon was probably enriched in refractory Ca and Al that allowed for early crystallization of feldspar. It is thus very unlikely that the Earth formed a similar early crust of feldspar. The lunar basalts, mostly erupted between 3,800 and 3,200 Myr, clearly preserve isotopic and trace-element evidence of being derived from a mineralogically zoned interior that had crystallized at 4,400 Myr. However, similar signatures are not evident in the earliest terrestrial rocks and conventional isotopic and trace-element evidence for crystallization of the expected early molten mantle is equivocal at the least.

So, was the terrestrial system so large that conventional wisdom, derived from layered intrusions on the Moon (two orders of magnitude smaller), is simply inadequate to describe the solidification of a molten terrestrial mantle? Indeed, alternative mechanisms of crystallizing

the mantle without producing separate mineral zones have been proposed⁵.

If the mantle had crystallized and formed an early crust (as did the Moon), the place to look for the evidence is in the oldest preserved Archaean rocks, such as the Acasta gneiss (3,960 Myr) and the famous exposures at Isua in Greenland, about 3,800 million years old, to see what evidence they preserve of earlier events. For some years, it has been apparent that some, although not all, early Archaean rocks appear to have been derived from an early mantle source that was depleted in light rare-earth elements (and hence indicative of the formation of an early crust enriched in light rare-earth elements). This evidence comes from the radioactive decay of ¹⁴⁷Sm to ¹⁴³Nd (with a geologically useful half-life of 106 billion years), and results in elevated ¹⁴³Nd/¹⁴⁴Nd ratios, or, in the jargon of the trade, positive $\epsilon_{143\text{Nd}}$ values. Nevertheless, such enrichments do not give unambiguous evidence of primordial differentiation; they may represent later differentiation events. Subsequent metamorphism or alteration might also produce such signatures.

However, isotope geochemists are a resourceful group, and as the sensitivity and precision of mass spectrometers have improved, new tests have become possible. Attention in the past year has become focused on a possible definitive resolution of this problem. In addition to the widely used ¹⁴⁷Sm–¹⁴³Nd system, samarium has another isotope, ¹⁴⁶Sm, which decays to ¹⁴²Nd with a geologically short half-life of 103 million years. ¹⁴⁶Sm is, of course, long since extinct, but it was around when the Solar System was young, a fact known from the presence of high ¹⁴²Nd/¹⁴⁴Nd ratios in meteorites⁶.

If similar ¹⁴²Nd excesses could be found in terrestrial samples, this would establish that differentiation processes did indeed occur on the Earth before 4,300 Myr, as ¹⁴⁶Sm was effectively extinct by that time. Such data might possibly settle once and for all the question of an early molten mantle and the formation of the long sought, but elusive, early crust⁷.

In this issue, Harper and Jacobsen¹, following earlier work, report a small enhancement (0.3 ϵ -units) in the ¹⁴²Nd/¹⁴⁴Nd ratio relative to terrestrial standards in a sample described as a meta-sediment from the 3,800-Myr supracrustal sequence at Isua, Greenland. Taken at face value, this would constitute evidence for early crustal differentiation. Harper and Jacobsen conclude that the combined evidence from the ¹⁴⁷Sm–¹⁴³Nd and ¹⁴⁶Sm–¹⁴²Nd decays suggests "that fractionation of Sm/Nd took place 4.44–4.54 Gyr ago, owing to extraction of a light-rare-earth-element-enriched primordial crust". No trace of

this crust has ever been identified.

However, their results remain equivocal, and questions have been raised at conferences. Other workers^{8,9} have been unable to find ¹⁴²Nd excesses in other Archaean rocks, including samples from the Isua, Akilia and Amitsoq gneisses from Greenland, the Barberton region in South Africa, the Pilbara of western Australia, and the currently oldest known terrestrial rock, the Acasta gneiss from the Northwest Territories of Canada, which is 150 million years older than the Isua sample examined by Harper and Jacobsen.

What do these conflicting measurements mean? Various explanations are possible. The Isua sample may be unique or aberrant, as other samples from that locality measured by other workers show no anomaly. In this context, a full description of the sample would be useful. Possibly it is telling us about some local sequence of events in a uniquely isolated segment of the mantle. It would clearly be useful for the other laboratories involved to repeat Harper and Jacobsen's analysis. The data reported by Harper and Jacobsen are close to the limits of precision obtainable from modern instruments.

One must also recall the many examples in science, ranging from super-heavy elements to martian canals, of problems in identifying phenomena close to the limits of resolution. Although it is always possible to obtain very high precision in the measurement of the ion beam in modern mass spectrometers, ultimately the reproducibility is governed by physical and chemical processes, often sample-dependent, operating during the production of the ion beam.

Clearly, further study of this apparently unique Isua sample in other laboratories is required before the exciting possibility of resolving one of the paradoxes of early Earth history can be pursued. Paradoxes, of course, arise not from the operations of nature, but from our imperfect understanding of them. □

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A molecular growth industry

Gary Ruvkun

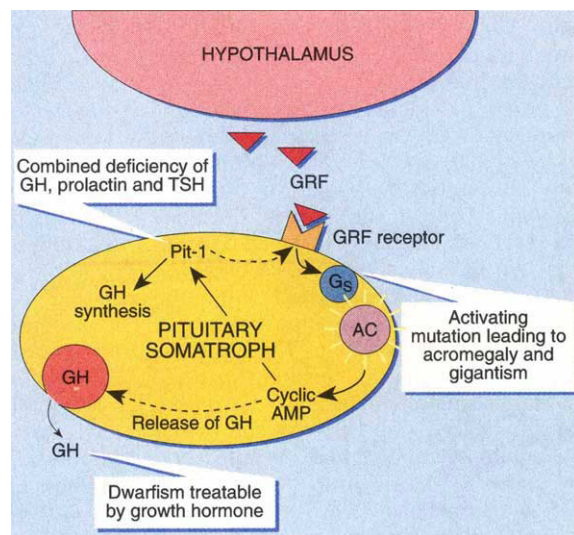
DWARFS and giants have been celebrated and feared throughout the ages, but recently they have figured large in establishing the molecular basis of the pituitary hormonal pathway that controls growth. Two new papers, by Lin *et al.* on page 765 of this issue¹, and by Mayo in a recent issue of *Molecular Endocrinology*² supply one of the missing links in this pathway, namely the receptor on the pituitary for the hypothalamic factor responsible for inducing the secretion of growth hormone (somatotropin) and the proliferation of the cells that secrete it.

The vertebrate pituitary gland produces growth hormone as well as other hormones that control the thyroid, adrenal glands and gonads. Each pituitary hormone or prohormone is synthesized by a distinct cell type whose proliferation and hormone secretion depends on continuous signalling from a corresponding releasing/proliferative hormone from the adjacent hypothalamus. Pituitary somatotroph cells are stimulated to proliferate and to secrete growth hormone by growth-hormone-releasing factor (GRF) from the hypothalamus. This factor couples to a signalling pathway in somatotrophs that uses cyclic AMP as a second messenger. Generation of somatotrophs as well as of two other pituitary cell types is dependent on the pituitary-specific transcription factor Pit-1. Many of the players in this signalling pathway are found to be mutated in particular dwarfism and gigantism syndromes (see figure).

Studies of GRF/somatotroph cell signalling have implicated a G_s-protein-linked receptor/adenylate cyclase pathway¹. Other neurotransmitters and peptide hormones that make use of this type of mechanism to convey information use a receptor that is a member of a superfamily of seven-helix transmembrane proteins. Lin *et al.* and Mayo therefore searched for a member that is expressed in the pituitary^{1,2}. Both found the same molecule, which has all the hallmarks of the same gene superfamily, and showed that it is expressed specifically in the pituitary of mice or rats. Each also found that when the receptor is expressed in non-pituitary cell lines, it activated cAMP synthesis upon addition of phy-

siological concentrations of GRF, but not of control peptides. Mayo was able to show specific binding of GRF at physiological concentrations to membranes of cells transfected with the cloned receptor. These data argue strongly that both groups have cloned a *bona fide* GRF receptor.

Lin *et al.* also show that expression of the GRF receptor is under the control of



Proposed interactions of Pit-1, the GRF receptor and synthesis of growth hormone (GH). Boxes show the effects of mutations in the components indicated. AC, adenylate cyclase; TSH, thyroid stimulating hormone.

the pituitary transcription factor Pit-1; neither GRF receptor nor growth hormone is expressed in a dwarf mouse bearing a putative null mutation in the Pit-1 transcription factor. The lack of GRF receptor expression in the dwarf mouse neatly explains the lack of somatotroph cell proliferation seen in the Pit-1 mutant: without GRF receptor, the somatotroph cannot receive proliferative cues from hypothalamic GRF. Still to be explained is why not only somatotrophs, but also lactotrophs and thyrotrophs, which secrete prolactin and thyroid-stimulating hormone respectively, also fail to proliferate in the dwarf mouse. Pit-1 may also activate the receptors for dopamine and thyroid-releasing hormone which are released from the hypothalamus to activate the proliferation of the corresponding pituitary cells. The dopamine D2 receptor is expressed in the pituitary³, and the dopamine agonists are used to treat pituitary tumours. One prediction is that these receptors are also not expressed in the Pit-1 mutant mouse.

Using the treasure trove of molecules that regulate and mediate the hypothalamic-pituitary-target-tissue signalling

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system, a number of dwarfism and gigantism syndromes in man have been explained by mutations in one of the encoding genes. For example, a point mutation just on the carboxy-terminal side of the Pit-1 homeodomain causes a dominantly acting human combined pituitary-hormone deficiency of growth hormone, prolactin and thyroid-stimulating hormone⁴, with a phenotype like that of the recessive Pit-1 mutant mouse. Consistent with its genetic dominance and similarity to the null phenotype, this mutant protein acts as an antimorph on testing for ability to transactivate a reporter gene while retaining the ability to bind DNA. Thus this mutation points to an amino-acid residue that can interfere with transactivation of downstream genes. Other less syndromic pituitary dwarfisms are caused by mutations in growth hormone itself, which can be treated with growth hormone injections, or in the growth hormone receptor, which cannot be treated in this way. So far, no pituitary dwarfism syndromes have been associated with mutations in GRF, though some have been treated with the hormone. There is some evidence that the molecule is expressed in the placenta as well as in the hypothalamus, and so might regulate other essential secretion and proliferation processes⁵.

Some giants have also been explained in terms of the molecules in this signalling pathway. GRF was originally identified from a pancreatic tumour that secreted high levels of GRF and caused proliferation of somatotroph cells and excessive secretion of growth hormone, leading to acromegaly (chronic growth of hands, head and feet); and overexpression of GRF in transgenic mice causes gigantism⁶. Some human pituitary tumours that lead to acromegaly are caused by a dominant activating mutation in a G_s α -subunit which constitutively activates adenylate cyclase to cause proliferation of pituitary cells. But the same mutations in this gene cause the more pleiotropic multiple endocrine neoplasms in McCune-Albright syndrome⁷. This G_s α -subunit must function in a variety of tissues so that the activating mutation deregulates a number of signalling pathways.

The cloning of the GRF receptor provides another reagent with which to search for genetic linkage to particular dwarfism or gigantism disorders. As the GRF receptor is specific to the pituitary, loss-of-function mutations in it should lead to non-syndromic dwarfism that is resistant to GRF treatment. The *little* mouse mutant is a candidate GRF-receptor mutant. The *little* mutant does not respond to GRF but does respond to growth hormone, suggesting that it is defective in hypothalamic signalling⁸. In

addition, the defect in growth hormone secretion can be bypassed with the adenylate-cyclase activator forskolin or the G_s activator cholera toxin, implying that the downstream signal transduction pathway is normal in this mutant. A key experiment is to show that the GRF receptor is closely linked to *little* and to look for allele-specific DNA sequence changes. Studies of human dwarfs showing similar responses to GRF and growth hormone should reveal loss-of-function mutations in the GRF receptor.

Dominant activating mutations in the GRF receptor would be expected to have the opposite phenotype, gigantism. Because the GRF receptor is a member of such a well-studied gene superfamily, an arsenal of results from yeast to man predict the function of GRF receptor domains and the consequences of mutations in those domains. For example, the carboxy-terminal cytoplasmic domain of the seven-helix transmembrane β -adrenergic and yeast α -factor receptors negatively regulates their coupling to G_s so that deletion of this domain causes hypersensitivity to their ligands^{9,10}. Similar mutations in this domain of the GRF receptor would be expected to cause hypersensitivity to GRF which could lead to somatotroph hyperplasia or tumours. In fact, there are pituitary tumours that do not carry mutations in the G_s α -subunit. However, a number of these pituitary tumours do not increase cAMP levels, suggesting that they are caused by mutations in protein kinase A which decouple it from its normal regulator cAMP, or act downstream of this point. Still, there is a strong pointer to the GRF receptor mutations that should be searched for in acromegalic tumours or in very tall people.

Given the dramatic progress in explaining and treating the underlying causes of dwarfism and gigantism, soon the endocrinologists will outnumber the dwarfs. They will keep busy, I predict, by studying the more subtle genetic variations in those of us whose height is fewer standard deviations from the mean, and of course by treating those who wish to move along the curve. □

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Cold light

DAEDALUS is surprised that astronomy works at all. We see the stars and galaxies through a thin veil of interstellar gas. It may be very tenuous, but surely it should blot out almost all distant objects by its sheer enormous thickness? If the enigmatic 'dark matter' of the Universe is also interstellar gas, the problem becomes even more severe. One possible escape is that interstellar gas is very cold. Most of it should be at 3 K, the background temperature of the Universe. Many conductors become superconducting at this temperature; by analogy, Daedalus expects that many insulators become super-transparent. They cease to interact electromagnetically, and their optical extinction coefficients go to zero.

This idea could easily be tested by cooling an optical fibre of known extinction coefficient in liquid helium. With good fortune, it would become super-transparent, so that light launched into it would travel for ever. A super-transparent fibre, closed into a ring and separated from its light source, should trap some light in endless circulation. Break it open some time later, and you should see a little flash as the trapped light escapes.

Such an effect could transform information technology. Supercooled optical fibre, needing no intermediate booster amplifiers, could greatly simplify telecommunications. A camera or photometer that did not merely record the effects of light, but trapped an actual sample of it for later study, could also be appealing. But the major implications are in optical computing. If light can be trapped in endless circulation, it could form the long-sought optical memory for such a computer. The crucial circuit element would be a circular loop of super-transparent fibre coupled to some sort of nonlinear optical switch. The switch would be triggered by a light pulse that altered its refractive index, flipping it briefly from total internal reflection to full transmission. The optical pulse train to be stored would enter the loop; by the time it had completed a circuit the switch would be reflecting again, and it would be trapped in continual circulation. The memory element could be interrogated at any time by flipping the switch again, thus recovering the stored pulse train. Unlike conventional semiconductor memories, a super-transparent memory would draw no power, and would continue to hold data when the computer was turned off.

Daedalus is now dreaming up other uses for stored light. He likes the idea of storing samples of summer sunlight for release in winter; but the volume of fibre required looks forbidding.

David Jones

Conventional wisdoms

Ziauddin Sardar

The Beginnings of Western Science: The European Scientific Tradition in Philosophical, Religious and Institutional Context, 600 BC to AD 1450. By David C. Lindberg. University of Chicago Press: 1992. Pp. 455. \$57, £45 (hbk); \$19.95, £15.95 (pbk).

The Struggle to Understand: A History of Human Wonder and Discovery. By Herbert C. Corben. Prometheus: 1992. Pp. 398. \$29.95, £18.95.

Is 'Western science', that is science as we know it today, purely a creation of Western civilization? Have other civilizations also played important roles in the creation and development of science? Or are all non-Western cultures simply bit players in the great 'history of human wonder and discovery'?

Before the recent revolutions in the history of Islamic, Chinese and Indian sciences, the conventional wisdom saw science and non-Western cultures as antonyms: science was the creation solely of Europe and almost totally the concern of Western civilization. Other civilizations and cultures had nothing to do with it. It began with the Greeks, was preserved during the 'dark ages' by the Arabs and finally 'transmitted' to its rightful owners at the dawn of the Renaissance. Thus the history of science was basically the history of Western civilization. But the picture has now changed considerably, as a casual perusal of the new edition of the *Dictionary of Scientific Biography* would confirm. Furthermore, as we learn more and more about science in non-Western cultures and civilizations, the conventional view appears not only untenable but frighteningly arrogant and Eurocentric.

The new research, however, has yet to make its impact on the general history-of-science surveys and texts, many of which — like de Santillana's *The Origins of Scientific Thought* (1961), Bernal's *Science in History* (1970) and Edward Grant's *Physical Sciences in the Middle Ages* (1971) — champion obsolete, conventional wisdom. The need for more up-to-date surveys of the ancient and mediaeval scientific world is self-evident. Lindberg's survey of *The Beginnings of Western Science*, the first in two decades, sets out to revise the record and offer a fresh synthesis of new information and interpretations.

The title of Lindberg's survey itself conveys the changing perceptions of science. He is concerned with the origins and development of 'Western science'; and not 'science' as some romantic, universal, ahistorical entity. Moreover, Lindberg's definition of science is very broad and incorporates the mediaeval notions of 'natural philosophy'. "We must respect", he notes, "the way earlier

generations approached nature, acknowledging that although it may differ from the modern way, it is nonetheless of interest because it is part of our intellectual ancestry." In recognition of Kuhn, Feyerabend and the sociology-of-knowledge school, Lindberg places ancient and mediaeval science in their philosophical, religious and institutional contexts. The overall accent of the survey is on the questions of continuity and transmission of scientific ideas from one civilization to the next.

But despite all his new data and interpretation, Lindberg's self-proclaimed "landmark" survey fails to rise above the Eurocentric perceptions of conventional history of science. We are taken on a roller-coaster ride from Babylonia and Egypt to Greece, Rome, Islam and mediaeval Europe. The underlying assumption is of a linear, darwinian ascent: Babylonia and Egypt are dismissed in five pages (Lindberg shows no awareness of the avalanche of recent material on the Afro-Asiatic roots of classical civilization; Martin Bernal's magnificent *Black Athena* (1987) does not even get a mention) and Islam is only there as a minor — but necessary in these days of political correctness — irritation. We are thus led to believe that 'Western science', the dominant mode of science in the world, is a European invention and a Western privilege.

For Lindberg, Islam is still a conveyor-belt civilization whose sole function in history is to preserve and transmit the Greek heritage to Europe. Indeed, many of his comments on the Muslim civilization are either blatantly ignorant or culturally biased. After a single-page caricature of the Islamic system of belief and thought, complete with almost every bogey of orientalist lore, Lindberg tells us that "the pursuit of knowledge for its own sake was never endorsed by Islamic religious ideology, nor by any other thread in the cultural fabric". It is rather surprising, then, that one-third of the Koran is devoted to asking the believers to pursue knowledge and describing the virtues of knowledge; that Prophet Mohammed could not praise knowledge highly enough; and that Muslim civilization produced more than 500 analytical definitions of knowledge! Lindberg tells

us further that "Muslims themselves divided learning into two categories: traditional, on the one hand; and foreign and 'rational'". The implication here is that even Muslims saw their traditional learning as irrational, so the rest of the world is right in seeing it as inferior. Lindberg's suggested Muslim division of learning is purely fictional: indeed it is an insult to the numerous intellectual giants such as al-Kindi, al-Farabi, ibn Sina and al-Ghazzali who produced extensive and detailed classifications of knowledge.

Lindberg discusses "the Islamic response to Greek science" by juxtaposing the so-called 'marginality thesis' with the 'appropriation thesis'. The marginality thesis was suggested in 1961 by the German orientalist G. E. von Grunebaum, who announced that "foreign sciences were marginal to Islam". The 'appropriation thesis' holds that Muslims appropriated Greek learning. Lindberg forgets to tell us that von Grunebaum's thesis has been totally discredited. Clearly, mathematics, astronomy, physics, chemistry, biology, botany, medicine, geography and other natural sciences were not foreign to Islam. And as far as Greek philosophy is concerned, it became the basis of one of the most powerful intellectual movements in mediaeval Islam, that of the Mutazalites; it was the Mutazalite philosophers such as al-Farabi, ibn Sina and ibn Rushd who introduced Europe to Plato and his disciples. Lindberg's discussion of the Muslim response to Greek philosophy is not only rooted in discredited orientalism, it would not even pass as an undergraduate essay.

The three pages devoted by Lindberg to "Islamic scientific achievements" are focused on a discussion of Pierre Duhem's 'challenge': "there is no Arabian [read 'Islamic'] science. The wise men of Mohammedanism were always the more or less faithful disciples of the Greeks, but were themselves destitute of all originality." Duhem made this statement in 1911. Some 80 years later, Lindberg is still struggling to make the colonial insult stick. Whatever happened to all that new research that is supposed to have changed our perception of Islamic science in recent decades? While a few notable historians of Islamic science appear in the bibliography, their work and thought is absent from the text.

Still, we have made some progress. Twenty years ago, Bernal dismissed Islam's contribution to Western science in 10 pages; Lindberg devotes 20 (including illustrations) to the same subject! But new research or not, the colonial perceptions of history of science are preserved in their banal totality.

To give him his due, Herbert Corben is very aware of the fact that an epic struggle of colonial proportions is being

fought in the history of science and ideas. *The Struggle to Understand* provides a popular counterpart to Lindberg's allegedly learned tome. Corben is very concerned with identifying common ground between Christians, Jews and Muslims. Indeed, he is so concerned with love and affection that he has thoughtfully provided a chapter listing all those wonderful men of classical civilization who suffered gallantly in the name of science. Words of prayer are said for Pythagoras, Socrates, ibn Sina, ibn Rushd, Galileo and a few others.

Unfortunately, sentiments are not enough for producing a worthy and "comprehensive history of scientific discovery and superstition from prehistoric time to the present". Corben seems to have culled his material from encyclopaedias, dictionaries and other tertiary sources. His description of the "magnificent contributions of Muslim scientists and physicians", for example, seems to

be based on a random selection of magazine articles. We thus get a very strange book, brimming with fine sentiments but which is bad history, bad analysis and badly written. Corben is a retired theoretical physicist; his book demonstrates that mastery of physics is not synonymous with intellectual acumen.

In his *New Organon* (1620), Francis Bacon set the agenda for all future histories of science by announcing that nothing worthy of attention existed between antiquity and him: "neither Arabians nor the Schoolmen need to be mentioned" for their science and thought is worthless. Nearly four hundred years on, and despite massive new research and politically correct sentiments, Bacon's spirit, as Lindberg and Corben illustrate so well, still permeates the historiography of science. □

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Ancient residents in resin

Paul Whalley

Life in Amber. By George O. Poinar Jr. Stanford University Press: 1992. Pp. 350. \$55.

IMAGINE being able to study an entire, three-dimensional fossil animal that was alive during the dinosaur era. It is so well preserved that you can also study its internal anatomy and extract and sequence DNA from its body tissue. Add to these advantages the fact that the fossil can easily be identified from only a nodding acquaintance with the present-day fauna and one would expect there to be a rush away from the impressions and flattened fragments usually studied by palaeontologists.

Perfectly preserved fossils of animals and plants, including fragile organisms such as insects, nematodes and fungi, are often found in amber, a fossilized plant resin. Plant resins preserve inclusions as they flow over them: with oxidation and polymerization, the resin becomes harder and forms a semifossilized product known as copal; after a few million years under the right conditions, copal becomes amber. Amber dating from more than 100 million years ago has been found, whereas Baltic amber, the commonest source for jewellery until the discovery of Dominican amber, is about 38 million years old. Resins are produced by a wide range of flowering plants and by conifers, and amber formation progresses in many parts of the world today: copal is still being produced by the Kauri pine (*Agathis australis*) in northern New Zealand.

Poinar discusses the formation of amber and copal and their chemistry, listing their sources and where the largest collections are now stored. He explains how to distinguish copal from amber and how to recognize fake amber containing present-day forms of life. When amber was merely a jewel, inclusions probably detracted from its value. Now, brilliant

coverage of this is very thorough and makes a real contribution to our knowledge. He considers the importance of amber in comparing recent and fossil faunas, pointing out that although fossil insect faunas show clear changes in composition after the Permian, there is little evidence of change at the Cretaceous/Tertiary boundary, despite the upheavals proposed by impact theories for the dinosaurs' disappearance.

An unanswered question is how the inclusions 'get inside' the amber in an apparently unruffled condition. It is difficult to imagine how insects are preserved with their delicate wings spread out inside a sticky resin. Perhaps insects are attracted to newly produced resin and then narcotized by another of its volatile substances. Maybe narcotized insects sink into the resin as gently as a specimen from xylene sinks into Canada balsam on a microscope slide preparation. But Poinar does discuss virtually all the other questions one may have about amber and its inclusions.

The 37 colour pictures in the book, mostly of Dominican amber, show just how perfectly preserved the specimens are. There are also more than 150 black-and-white photographs interspersed throughout the text, and about 30 pages of references. (I was therefore surprised at the omission of Helen Fraquet's book on amber published in the Butterworth Gem series in 1987.)

One main problem with insects in amber is establishing their exact taxonomic identity. The insects are so similar to living species that many nontaxonomists have been tempted to name them. For example, Poinar reproduces a photograph of 120-million-year-old Lebanese amber that I supplied, containing a Psychodid fly that I am assured differs from modern ones only in wing-venation, a difference unrecognizable except by a taxonomist familiar with the group. Sadly, as Poinar remarks, "entomologists tend to shunt amber inclusions to paleontologists, who for the most part, are concerned with studying groups in the fossil record that have no direct descendants today". He points out that "there has been a striking decline in the number of organismic biologists, especially taxonomic systematists, during the past two decades".

Poinar considers the implications of amber for biological science and believes that there are tremendous possibilities for future work, from biogeography and reconstructing ancient landscapes to tissue preservation. His comprehensive and readable reference book should greatly benefit these studies. □

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Sticky end — a soldier fly (family Stratiomyidae) in Dominican amber.

jewels containing, for example, a pristine 30-million-year-old ant, fetch an ever-increasing price.

Much of the text is devoted to a systematic review of the plant and animal inclusions found in amber. Poinar is a specialist on Dominican amber and

From *Life in Amber*

Formation of dwarf galaxies in tidal tails

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ZWICKY¹ argued long ago that tidal forces can tear long tails of stars and gas from the bodies of interacting disk galaxies, and that this debris may include self-gravitating objects which could become small galaxies. Some recent observations revealing small clumps of stars and gas in tidal tails lend weight to this idea²⁻⁴. Here we report the results of numerical simulations of encounters between disk galaxies⁵, each modelled with a central bulge, an exponential disk and a spheroidal dark-matter halo. We find that dwarf systems form in material drawn out during the encounter; these objects can capture large amounts of moderately enriched gas, but retain little dark matter from their parents' haloes. They should therefore have lower mass-to-light ratios than galaxies formed directly by the collapse of primordial material.

Over the past two decades it has become clear that tidal forces between disk galaxies can extract ribbon-like 'bridges and tails'; still controversial is the conjecture that disk galaxies can merge to form elliptical galaxies⁶. One counter-argument to this merger hypothesis is that dwarf elliptical galaxies could not have formed by merging because no smaller progenitors exist. Some dwarf galaxies may, however, form in collisions between giant systems.

The first example reported¹ was the diffuse cloud found at the end of the southern tail of NGC4038/9. Schweizer² noted that this object contains emission-line nebulae, and Mirabel *et al.*³ showed that the stars powering the nebulae are considerably younger than the tails themselves and therefore formed *in situ*. The total luminosity, hydrogen mass and dynamical mass of this object seem typical of dwarf irregular galaxies. Similar clumps, although more compact, are seen in both tails of the well known merger remnant NGC7252 (ref. 2). Recent 21-cm observations reveal that these also contain substantial amounts of neutral hydrogen (J. Hibbard and J. van Gorkom, personal communication). An even more striking example is the infrared-luminous system IRAS19254-7245 (refs 1, 7). These two gas-rich galaxies sport a remarkable pair of tidal tails several hundred kiloparsecs long; distributed along these tails are at least nine distinct knots or condensations of luminosity. The broadband $B - V$ colours of these knots range between 0.4 and 1.1, suggesting that some are sites of star formation⁴.

Such self-gravitating structures cannot form in test-particle models of interacting disk galaxies⁶, and self-consistent models with $\leq 10^3$ particles⁸ lack the mass resolution to reproduce them. But bound clumps in tidal tails are found in self-consistent models with $\sim 10^5$ particles, such as the one we describe here.

We begin by building galaxy models⁹, each containing a central bulge, an exponential disk and a spheroidal halo, with mass ratios of 1:3:16, respectively. We use arbitrary units with gravitational constant $G \equiv 1$; in these units, each galaxy has total mass $M = 1.25$, half-mass radius $R_{1/2} \approx 0.25$, and binding energy $E \approx -1.38$. These model galaxies may be roughly scaled to the Milky Way by equating our units of length, mass and time to 40 kpc, 2.2×10^{11} solar masses $M_\odot$ and 250 Myr, respectively.

Two such galaxies are released on parabolic orbits chosen to reach a pericentric separation of 0.2 length units one time unit after the calculation starts. One disk lies exactly in the orbital plane and spins in the same direction as the galaxies orbit each other; the other disk is inclined by 71° from the orbital plane, with its line of nodes turned by 30° from the separation vector at pericentre in the direction opposite the orbit.

This encounter is simulated with a combined N -body/smoothed particle hydrodynamics (SPH) code¹⁰ using 90,112 particles, of which 16,384 represent gas. The gas, which is 10% of the disk mass, is initially distributed just like the disk particles. Radiative cooling is included using a solar-abundance cooling curve, cut off below 10^4 K to suppress local instabilities. A standard form of artificial viscosity numerically broadens shocks.

The dynamics of this encounter are described elsewhere⁵, so they are only summarized here. Following their first passage, both galaxies develop extended tidal features. Tidal friction between the extended haloes causes the relative orbit to decay rapidly. A second, nearly head-on passage occurs ~ 1.5 time units after the first passage, and the galaxies coalesce ≤ 0.5 time units thereafter. At later times the tails expand kinematically while the merger remnant becomes more regular through phase-mixing.

Bound structures develop in the tails even before the galaxies merge, and they become more conspicuous as the tails spread out. Figure 1 shows the merger remnant 3 time units after the calculation starts and an enlarged view of the most massive dwarf in the tail from the in-plane disk. This object has a mass of 0.0019 units and contains ~ 360 particles; its half-mass radius is ~ 0.019 length units and its instantaneous tidal radius is nearly five times greater. Gas constitutes $\sim 25\%$ of the mass and is more centrally concentrated than the stars; this gas was accreted from material compressed into a thin 'ridge-line' along the tail by global shocks¹¹. Dark matter from the haloes of the original galaxies, in contrast, comprises $< 5\%$ of the dwarf's mass.

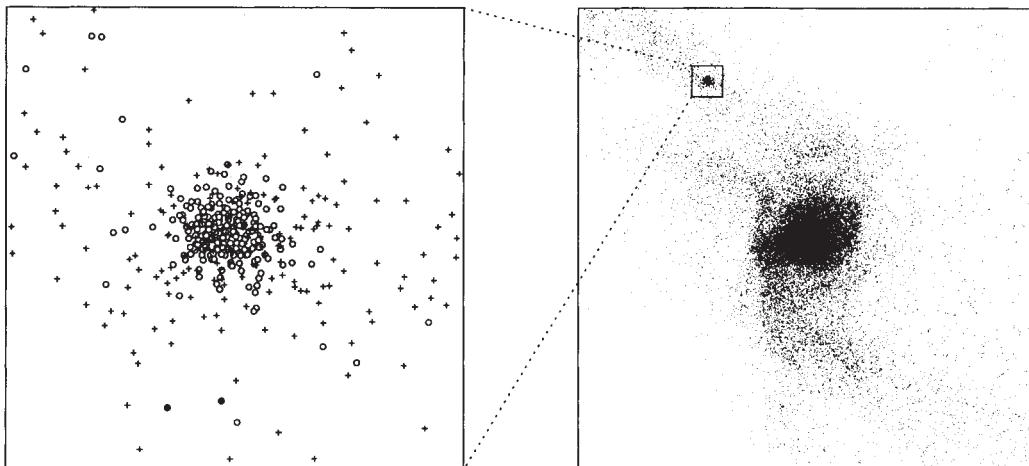
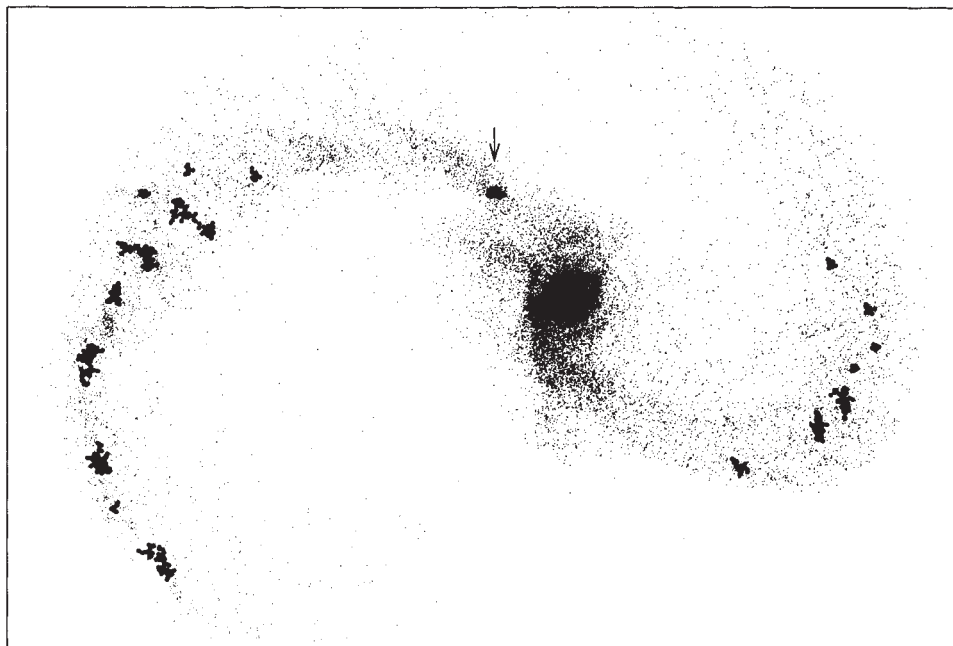


FIG. 1 (Right) Remnant produced by the merger of two disk galaxies, 3 time units after the simulation starts. The field is 2.4×2.4 length units. Only particles from the disks and bulges of the original galaxies are shown. (Left) Close-up of the most massive dwarf object produced in this simulation; the field is 0.16×0.16 units. Gas particles are plotted as open circles, stellar particles as crosses and dark-matter particles as filled circles.

FIG. 2 Distribution of bound objects in the merger remnant, 3 time units after the simulation starts. The field is 7.2×4.8 units. Only particles from the disks and bulges of the original galaxies are shown. Particles belonging to bound objects are plotted as filled circles. The arrow indicates the most massive dwarf.



We use a 'friends-of-friends' algorithm to systematically find other bound objects in the tidal tails; two particles are members of the same clump if they lie within a distance b of each other or can be linked by any number of particles each lying within a distance b of the next. As some clumps are much more diffuse than others, we combine results for b values between 0.01 and 0.04 length units. Most of the objects in the final catalogue have kinetic to potential energy ratios $T/U \approx -0.5 \pm 0.1$.

Figure 2 shows the 23 objects containing 12 or more particles each identified in this simulation. Unlike the massive object shown in Fig. 1, the other objects are gas-poor. To retain much gas, an object's virial temperature T_{vir} must be greater than the cooling cut-off temperature of 10^4 K. We computed virial temperatures from mean-squared velocity dispersions; the most massive and gas-rich object has a temperature $T_{\text{vir}} \approx 26,000$ K, whereas the next largest, which is gas-poor, has $T_{\text{vir}} \approx 5,000$ K.

As Fig. 2 illustrates, the less massive objects are confined to the outer tidal tails. Most fit comfortably within their present tidal radii, but tidal forces probably stopped low-mass objects closer to the merger remnant from collapsing significantly. The most massive objects are more resistant to tides and hence not confined to the outer regions; in this experiment the most massive object formed close to the remnant, but in a pure N -body version of this encounter⁹ the most massive object formed considerably further out. The figure also shows that the tail from the inclined disk produces fewer objects than the tail from the in-plane disk. We do not fully understand why this is so, although the inclined tail is more diffuse to shear forces during the passage.

The integrated properties of these simulated objects are consistent with those of some dwarf galaxies. The most massive objects reach $\sim 4 \times 10^8 M_{\odot}$ and have projected surface densities $\geq 10^2 M_{\odot} \text{ pc}^{-2}$; combining high gas contents with substantial intermediate-age stellar populations, they might well pass for dwarf irregulars. Less massive objects, which have lower surface densities and lack the raw materials for star formation, may come to resemble dwarf spheroidal galaxies. In either case, mass-to-light ratios should be low, as these objects capture very little collisionless dark matter from the haloes of their parent galaxies. Data on mass-to-light ratios of dwarf galaxies are scarce, but some dwarf spheroidals have mass-to-light ratios of order unity¹² as expected for systems with little dark matter.

Nonetheless, the relation between the objects found in these models and the knots seen in the tails of real galaxies remains obscure. Particle noise, boosted by swing amplification¹³ in the pre-encounter disks, appears to provide the density fluctuations which collapse to form bound objects in our simulations. The origins of density fluctuations in real tidal tails are unclear. Thus although our simulations show how gravitational instability can produce dwarfs, they may not correctly predict the distribution of masses or binding energies of such objects. An analogy may be made with cosmological simulations; for example, models starting from white-noise initial conditions¹⁴ illustrate gravitational clustering but do not reproduce the spectrum of objects obtained from more cosmologically plausible initial conditions¹⁵.

The general idea that dwarf galaxies could form by gravitational collapse in expanding positive-energy stellar systems, including tidal tails, was previously advanced by Gerola *et al.*¹⁶ A slightly different argument is made by Elmegreen *et al.*¹⁷; although also emphasizing gravitational instability, they propose that agitation of gas in interacting galaxies increases the Jeans mass to $10^8 M_{\odot}$, the mass scale of the resulting dwarfs. In our models, bound objects form as a result of gravitational collapse in the stellar component, with gas collecting in the deepest potential wells afterwards.

Gravitational collapse in itself does not explain why dwarfs are found so near the ends of the stellar tails in both NGC4038/9 and NGC7252. Mirabel *et al.*³ suggest that such dwarfs might arise from gas distributed beyond the stellar disks of the pre-encounter galaxies; they also raise the possibility that gas in tails may be compressed to the point of collapse by hot intergalactic gas. In NGC7252, the gas tails extend to almost twice the lengths of the stellar tails (J. Hibbard and J. van Gorkom, personal communication), in accord with the first suggestion. But there is no sign that the gas is interacting with a much hotter medium; the good alignment between the stellar and gas tails suggests that both are expanding freely. Further examples of dwarfs at the ends of optical tails might force a re-examination of this question.

Our results suggest that not all galaxies formed from primordial perturbations; some dwarfs may be secondary objects torn from larger galaxies by tidal forces. Such dwarfs should have relatively low mass-to-light ratios; they may also have unusual metal abundances, as they formed from material first processed

in the deeper potential wells of large galaxies². Further studies may lead to the identification of such galaxies as a distinct class of dwarf systems. □

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Three-dimensional image of the solar corona from white-light observations of the 1991 eclipse

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THE structure of the solar corona is believed to be governed by the solar magnetic field induced by currents at the surface of the Sun and perhaps within the corona itself¹. Inhomogeneities in the corona are well known from radially compensated eclipse images² in white light or coronal emission lines³. We have used two white-light images taken about three hours apart by the Multi-station International Coronal Experiment⁴ during the July 1991 total eclipse in an attempt to deduce directly the three-dimensional structure of the corona. We observed prominent coronal structures, including broad threads, rays and streamers, and used them to calibrate a model based on solid-body rotation and bulk coronal outflow. The errors in the reconstruction method are small enough to give us confidence that quasi-rigid rotation is a reasonable approximation over these timescales. We illustrate the deduced coronal structure by means of a stereogram.

As the typical coronal temperature is 2×10^6 K and the coronal plasma is therefore fully ionized, density inhomogeneities show up in white-light images of the eclipse thanks to Thomson scattering of the Sun's light from free electrons. At equal radial distances, pressure differences observed at the edges of a structure must be balanced by the local magnetic field pressure; the field has not yet been carried out by the wind to the intermediate corona. The origin of the coronal magnetic field is not well understood.

Attempts have been made to infer the structure of coronal magnetic field lines from the observed line-of-sight component of the magnetic field⁵ in photospheric layers. In these attempts, currents have been ignored although twisted structures are observed everywhere, and, more important, the canopy field observed under the corona has also been ignored. Furthermore, it is not clear what fields in the photosphere or the chromosphere are important when comparing coronal structures: those from

sunspots⁶, those from the edges of coronal holes⁷ or those associated with prominent bright regions and filaments^{8–10}. The research so far has shown the importance of coronal holes (with no photospheric counterparts) especially in polar regions, and of the equatorial 'belt of magnetic activity' which seems representative of the coronal structure responsible for the three-dimensional helio-sheet¹¹, the layer of interplanetary plasma surrounding the Sun at different latitudes⁵.

When analysing coronal structure, one is immediately faced with the question of interpreting a three-dimensional distribution of structures projected on the plane of the sky, which is what we observe in white light during eclipses. The white-light corona is optically thin and polarized. The polarization ratio, which ought to be helpful, is in fact ambiguous¹². Images of the corona have been obtained at many points in the Sun's rotation by using satellite-borne externally occulted coronagraphs, and an attempt was made to use the rotation to deduce the three-dimensional structure of the June 1973 corona¹³. This did not work well for several reasons: (1) the spatial resolution was too poor to measure small shifts; (2) shadows cast by the pylons supporting the occulting discs obstructed the image; (3) observations made on different days are affected by intrinsic coronal changes due to dynamical effects; (4) data could not be obtained in the most interesting region, between the surface and 1 solar radius ($R_\odot$) from the surface, because of the externally occulting out-of-focus discs. Snapshots taken during total eclipses avoid these defects. Data can only be collected, however, during the short time for which the Moon's shadow crosses the Earth (typically a few hours) so the time lapse covered is small.

A possible objection is that, as in point (3) above, the flow responsible for the solar wind could affect results. But in fact this flow is mainly directed outwards (along the structure), whereas we consider here effects produced by the quasi-rigid rotation. Moreover, it has not been demonstrated that solar wind flow occurs in dense coronal structures; downflows cannot be excluded. In this respect, our approach might provide some insight into the source of the solar wind.

Coronal structures are evident when using radial neutral filters on high-contrast prints (see Fig. 6.1 of ref. 2). Such pictures were obtained by the Multi-station International Coronal Experiment⁴ run during the 11 July 1991 total eclipse from sites at Hawaii, Mexico and Brazil. Structures are seen on these pictures⁴ where large transverse gradients occur, and may be in the form of jets, broad threads or streams and, especially, long streamers. Here we use the general term 'ray' to describe these structures. Contours outlining them are easily drawn, once the monotonic radial gradient has been removed.

For two pictures obtained at different times, we now consider shifts in the positions of structures, measured orthogonally to radial drawn on an enlarged print. We define ω as the angular velocity of (rigid) rotation of the Sun; $\mathbf{r}$ as the radius vector from the Sun's centre to the point (x, y, z) , where the y -axis extends along the direction of ω ; $\boldsymbol{\tau}$ as a unit vector from the Sun towards the observer; $\mathbf{l}$ as a unit vector along the coronal structure being considered; and $\mathbf{v}_c$ as the intrinsic bulk velocity of the coronal plasma.

Spatial shifts of point $\mathbf{r} = \{x, y, z\}$ measured in projection on the plane of the sky after a time lapse δt can be written as

$$\boldsymbol{\xi} = (\boldsymbol{\omega} \times \mathbf{r} + \mathbf{v}_c) \delta t \quad (1)$$

If we consider only components parallel to the plane of the sky this becomes

$$\xi_{\parallel} = \boldsymbol{\xi} \cdot \boldsymbol{\tau} (\boldsymbol{\xi} \cdot \boldsymbol{\tau}) \quad (2)$$

We cannot actually measure the full $\xi_{\parallel}$ but only its part orthogonal to the structure, in the plane of the sky

$$\xi_R = \xi_{\parallel} - \mathbf{l}(\boldsymbol{\xi} \cdot \mathbf{l}) \quad (3)$$

Writing equation (2) as $\boldsymbol{\tau} \times (\boldsymbol{\xi} \times \boldsymbol{\tau})$ and the equivalent expression

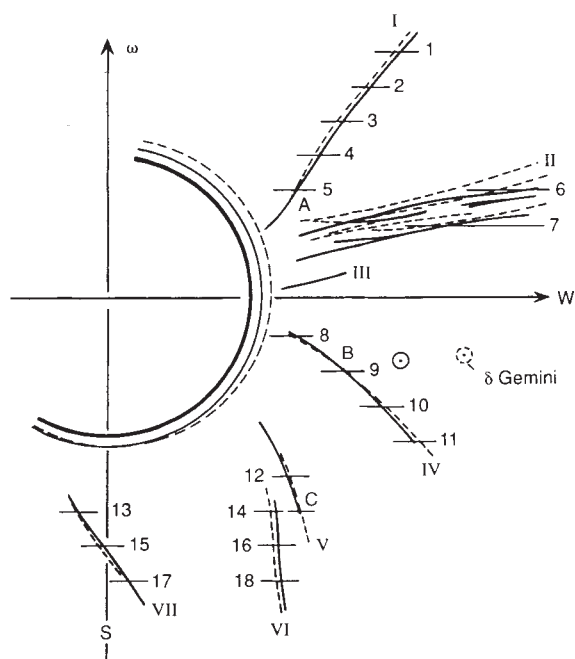


FIG. 1 Structures at the west limb of the eclipse corona of 11 July 1991, as deduced⁴ by analysing two radially filtered pictures obtained at more than 3 hours interval. Full lines, as observed at Hawaii; dotted lines, as observed at Tefe (Brazil) 3 h 13 min later. Locations over the coronal picture where measurements of shifts z in solar radii are made (see equation (7)) are numbered.

Number	1	2	3	4	5	6	7	8	9
z	1.8	1.5	1.1	1.2	0.2	19	15	1.5	0.08
Number	10	11	12	13	14	15	16	17	18
z	-0.86	-1.9	-0.31	0.23	1.1	0.7	0.93	0.08	0.93

for equation (3), we can rewrite equations (1)–(3) as

$$\xi_R = \mathbf{I} \times \{[\boldsymbol{\tau} \times \{(\boldsymbol{\omega} \times \mathbf{r} + \mathbf{v}_c) \times \boldsymbol{\tau}\}] \times \mathbf{I}\} \delta t \quad (4)$$

At $r = 2R_\odot$, $|\boldsymbol{\omega} \times \mathbf{r}| = 4 \text{ km s}^{-1}$; we suspect that bulk velocities in the corona may exceed 4 km s^{-1} and would like to take those velocities into account. However, their transverse component is certainly of smaller amplitude, at least in the quiet corona, so we will assume here that $|\mathbf{v}_c| \ll |\boldsymbol{\omega} \times \mathbf{r}|$ and neglect $|\mathbf{v}_c|$ from now on. Using equation (4), we rewrite shifts of coronal structures as

$$\xi_R / \delta t = (\mathbf{I} \times \mathbf{r})(\boldsymbol{\omega} \cdot \boldsymbol{\tau})(\mathbf{I} \cdot \boldsymbol{\tau}) - (\mathbf{I} \times \boldsymbol{\omega})(\mathbf{r} \cdot \boldsymbol{\tau})(\mathbf{I} \cdot \boldsymbol{\tau}) + (\mathbf{I} \times \boldsymbol{\tau})\{(\mathbf{I} \cdot \boldsymbol{\omega})(\mathbf{r} \cdot \boldsymbol{\tau}) - (\mathbf{I} \cdot \mathbf{r})(\boldsymbol{\omega} \cdot \boldsymbol{\tau})\} \quad (5)$$

Equation (5) shows that $\mathbf{I}$ can effectively be defined as a unit vector in the plane of the sky; in that way $\mathbf{I}$ is related to the observed phenomena. Equation (5) can then be simplified by dropping terms in $(\mathbf{I} \cdot \boldsymbol{\tau})$. A second simplifying assumption that we adopt is to consider the axis of solar rotation as orthogonal to the line of sight. Its amplitude reaches a maximum value of

$90^\circ \pm 7^\circ$, and on 11 July 1991 it was 86° . When $(\boldsymbol{\omega} \cdot \boldsymbol{\tau}) = 0$, equation (5) becomes

$$\xi_R = (\mathbf{I} \times \boldsymbol{\tau})(\mathbf{I} \cdot \boldsymbol{\omega})(\mathbf{r} \cdot \boldsymbol{\tau}) \delta t \quad (6)$$

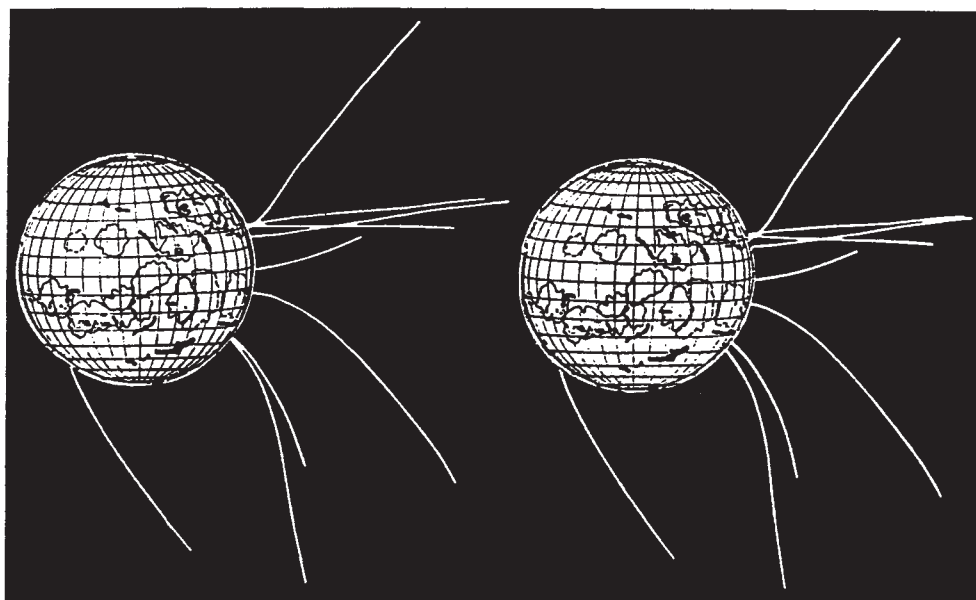
Obviously $\mathbf{I} \times \boldsymbol{\tau} = \mathbf{i}$ where $\mathbf{i}$ is the unit vector orthogonal to the ray at any point but in the plane of the sky (the plane $z = 0$). Then $(\mathbf{r} \cdot \boldsymbol{\tau}) = z$ and finally, denoting the angle between $\boldsymbol{\omega}$ and $\mathbf{I}$ as γ (Fig. 1), we rewrite equation (6) as

$$\xi_R = \mathbf{i} \omega \delta t z \cos \gamma \quad (7)$$

Let us now consider Fig. 1. Because none of the rays present are strictly parallel to the equator when $(\boldsymbol{\omega} \cdot \mathbf{I}) = 0$, then a zero shift should correspond to $(\boldsymbol{\tau} \cdot \mathbf{r}) = 0$ as clear from equation (6), or if $z = 0$ in equation (7). Three such points are labelled A, B and C on Fig. 1. These points indicate where rays cross the plane of the sky through the solar centre and give the first indication of the three-dimensional structure of the corona.

This limited information can be substantiated by looking more carefully at Fig. 1. As an example, let us consider ray IV. In

FIG. 2 Stereo diagram constructed using data given in Fig. 1 and data on solar disc activity on 11 July 1991 from the Russian 'Soln. Dannye' of the Russian Academy of Science (Solar Data).



equation (7), given the measured shift ξ_R , we wish to find the value of z in $|\omega| \delta t z \cos \gamma$. For the rotation period, we take $T = 27$ days. The daily angle of rotation is $|\omega| \delta t \approx 13^\circ$, so for $z = 1 R_\odot$, $|\xi_R| \approx R_\odot/4$; on Fig. 1, the time interval $\delta t \approx 3$ hours (3 h 13 min for pictures taken near the middle of totality at Hawaii and Tefe; Zirker *et al.*⁴), so $|\xi_R| = R_\odot/30 \approx 30$ arcsec and this shift increases linearly with z .

Ray VI in Fig. 1 is quasi-parallel to ω . We measured a shift of $0.04 R_\odot$, so its distance from the plane of the sky should be $1.5 R_\odot$; the ray seems to be parallel to this plane. We rewrite equation (7) in a more convenient form using $\delta\varphi = \omega \delta t$ and note that

$$\delta x = \xi_R / \cos \gamma \quad (8)$$

where δx is the shift of the ray along the x -axis. Then equation (7) becomes

$$z = \delta x / \delta\varphi \quad (9)$$

Because $\delta\varphi$ is constant for a rigid rotation, z should be proportional to δx .

On Fig. 1 we show the position of horizontal cuts used to determine the values of z shown in the table, and to define the three-dimensional structures considered. We used those parameters to assemble the stereo image shown in Fig. 2. Looking at Fig. 2, we clearly see ray I coming toward the observer. The system of rays II shows large variations for which an explanation has been offered previously⁴. If we attribute these to three-dimensional considerations and rotation alone, we deduce large δx and thus large z (deviations from the plane of the sky); in that case the system of rays II would be directed towards the observer, which is possible, but unlikely. In addition, the solar

region under system II was very active, so we prefer the interpretation given in ref. 4 in term of true intrinsic variations of structures. Ray III did not change its position so it is in the plane of the sky. Ray IV comes from the region outside the plane of the sky, crosses it at point (2) and then is directed towards the observer; ray V is similar but barely crosses the plane of the sky. Rays VI and VII have a similar direction, almost parallel to the plane of the sky, and lie in the region $z > 0$.

For more careful analysis of the structure of the corona, several improvements could be made to our method. First, the polarization ratio of white light from structures should be used¹², as this is a function of z and r . Second, results from the theory of continuous mapping of surfaces (theory of 'catastrophes') could be introduced. Such improvements would improve the precision of the analysis and might fully resolve the old problem of the three-dimensional structure of the corona. $\square$

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Evidence of a transition temperature for the optimum deposition of grafted monolayer coatings

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TECHNIQUES for surface modification are of considerable technological interest for the fabrication of water-repellent and anti-fouling coatings. Silanization¹ (the chemical grafting of organic molecules onto a substrate via a trichlorosilane group) stands out among these techniques by virtue of its ability to provide highly compact coatings of optical quality, extreme chemical inertness and adjustable wettability². Although the silanization reaction has been extensively characterized^{3–8}, the properties of the grafted layers are still too variable for most commercial applications; for example, the quality of the grafted layers depends critically on the presence of trace amounts of water, and on the temperature at which the silanization reaction takes place⁹. Here we provide evidence for the existence of a near-ambient temperature threshold, T_c , which represents an upper bound for obtaining the highest-quality films. This threshold temperature is found to be an intrinsic property of the silane molecules: it depends linearly on their chain length, but is independent of the solvent used for the reaction. We suggest that T_c is analogous to the triple point in the phase diagram of Langmuir monolayers.

Figure 1 shows a step-by-step protocol for the silanization of oxidized silicon wafers. The first six stages are to ensure the extreme cleanliness of the substrate, and especially to remove

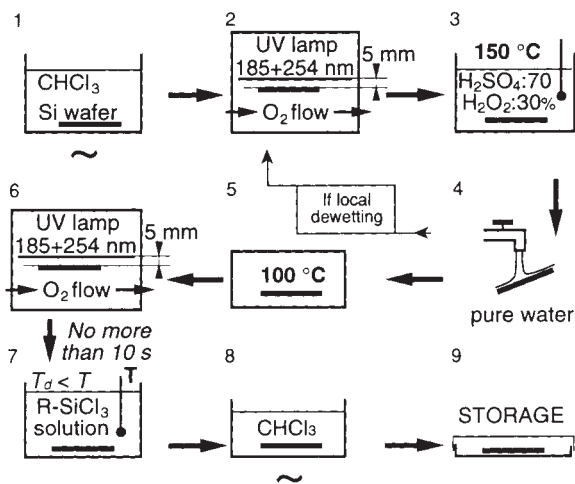


FIG. 1 Silanization process, including the six-stage procedure for cleaning the solid substrate. (1) Degreasing by sonication for 3 min in two successive chloroform baths. (2) Dry cleaning of both sides of the substrate by ultraviolet/O₃ photo-oxidation until the substrate becomes completely wettable by water. (3) 'Wet' chemical attack in freshly prepared acid mixture (5 min). (4) Careful rinsing (running tap, distilled and Millipore water successively). (5) Drying in still air, as short as possible. (6) Final cleaning of the front surface by ultraviolet/O₃ photo-oxidation (30 min). (7) Immediate silanization for 10 min at controlled T_d and dewpoint T_d , in a freshly prepared mixture of alkane (70%), carbon tetrachloride (~30%) and 10^{-3} M of *n*-alkyl-trichlorosilane (added less than 1 min before dipping the wafer). (8) Sonication (3 min) in two successive chloroform baths to remove the unreacted compounds. (9) Storage of the grafted substrate in a desiccator: no special care is necessary.

contamination by surfactants. Both wet cleaning with organic and aqueous chemicals, and dry cleaning by combining ultraviolet irradiation and chemical oxidation in an ozone atmosphere¹⁰, were used before starting the silanization itself. Water uptake

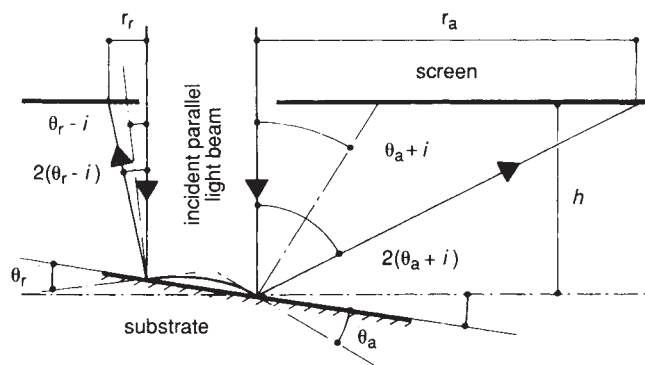


FIG. 2 Method for measuring simultaneously the advancing (θ_a) and receding (θ_r) contact angles, adapted from a recent optical technique¹⁵. The droplet acts like a convex mirror for a parallel light beam, the shape of the reflected beam giving the contact angle. The substrate is tilted until the drop starts to move by gravity¹⁶. If the velocity is small enough, the profile is quasistatic¹⁷, and the dynamic contact angles are identical to their static counterparts; moreover, the uncertainties due to inertial effects when depositing a drop of low viscosity on the substrate are avoided. The angles are given by $\tan 2(\theta_r - i) = r_r/h$ and $\tan 2(\theta_a + i) = r_a/h$, where i is the tilt angle of the plate, and r_a and r_r are the radii of curvature on the slip axis of the drop, forward and backward respectively. For small angles, the accuracy on the measured angle is better than $\pm 0.5^\circ$.

by the solution is also minimized. Trichlorosilanes are prone to self-polymerization² and this manifests itself as flakes in the solution or as a thin haze at the liquid surface. Therefore, atmospheric humidity was controlled to ensure that the dewpoint T_d was lower than the reaction temperature. On the other hand, in accordance with other reports⁹, we did not attempt to remove the water physisorbed on the silicon wafers as this is suspected to play a role in the physisorption of the reagent molecules. We therefore baked the substrates at moderate temperatures, $\sim 100^\circ\text{C}$ (ref. 11). Details of the reaction will be given elsewhere. Here it suffices to say that the temperature of the reaction was systematically varied between 0 and 60°C .

The quality of the grafted alkyl-trichlorosilane layers was checked by measuring the critical surface tension γ_c of the modified substrates. This was done by Zisman's extrapolation method¹² in which the contact angles for sessile drops of a

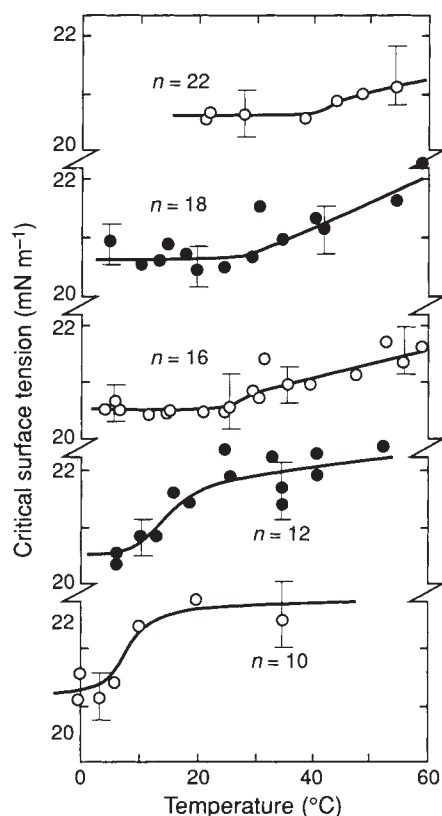


FIG. 3 Variation of the critical surface tension γ_c of silicon wafers covered by n -alkyltrichlorosilane monolayers as a function of the temperature of the silanization reaction. The different symbols correspond to alkylsilanes with different chain lengths ($n=10, 12, 16, 18, 22$ from top to bottom). At low enough temperatures, a constant γ_c value of $20 \pm 0.5 \text{ mN m}^{-1}$ is reached, independent of n . Note the systematic shift, with increasing n , of the transition temperature T_c separating the high-temperature from the low-temperature behaviour.

homologous series of apolar liquids are plotted as a function of their liquid-vapour interfacial tension γ_{LV} . The experimental set-up used to measure the contact angles is shown in Fig. 2. Both advancing and receding contact angles, θ_a and θ_r , can be obtained simultaneously by tilting the substrate during the measurement.

Three test liquids (n -octane, nonane and decane) were selected, and the data points shown in Fig. 3 indicate a linear relationship between $\cos \theta_a$ and γ_{LV} . Extrapolation to $\cos \theta_a = 1$ yields the critical surface tension to an accuracy of $\pm 0.5 \text{ mN m}^{-1}$. The choice of low-molecular-weight alkanes with γ_{LV} close to γ_c helps, as it guarantees small contact angles (for which our measuring technique is more accurate) and makes extrapolation to θ_a less uncertain. The γ_c values measured for wafers prepared at different temperatures T have been collected in Fig. 3. The solid lines are just guides to the eye, and each one corresponds to an alkyl-trichlorosilane with a different chain length ($n=10$ – 22). There is a clear dependence of γ_c on T , and one can distinguish two regions. Below a temperature threshold, T_c , γ_c has a low and constant value of $20.5 \pm 0.5 \text{ mN m}^{-1}$. Moreover, it is independent of alkyl chain length. This value is characteristic of dense packing of methyl-terminated hydrocarbon chains and agrees well with recent results obtained elsewhere^{3,8,9}. In refs 3 and 8, the densest monolayers are characterized by an advancing contact angle of 45° for a sessile drop of n -hexadecane, in accordance with our own observation. Above T_c , in contrast, γ_c increases rapidly with temperature. As γ_c for polyethylene chains is $\sim 31 \text{ mN m}^{-1}$ (ref. 12), the observation of a γ_c value intermediate between 20.5 and 32 is consistent with a disorganized layer containing a mixture of $-\text{CH}_2-$ and $-\text{CH}_3$ groups at the air-monolayer interface. This degradation of the layer packing has been confirmed by preliminary ellipsometric measurements (A. N. Parikh and D. Allara, personal communication) which show a 20% loss in the grafting density when the silanization is done at high temperatures.

The variation of T_c with chain length n is given in Fig. 4. We have estimated T_c empirically as the intercept in Fig. 3 between the horizontal asymptote and a line passing through the γ_c values in the rapidly increasing region. We estimate the accuracy of T_c to be $\pm 1.5^\circ\text{C}$. It is striking to observe that T_c increases linearly with chain length between $n=10$ and $n=22$, shifting by $3.5 \pm 0.5^\circ\text{C}$ for each additional methylene group in the grafted chain. These threshold temperatures do not depend on the nature of the solvent used in the silanization reaction. Identical results were obtained with n -decyl-trichlorosilane dissolved in decane, dodecane or iso-octane (a branched chain). T_c is therefore an intrinsic property of the alkyl-trichlorosilane to be grafted. Our experiments do not support the assumption that the influence of the reaction temperature on the final quality of the monolayer is related to differences in the solubility of the silane in the reactive solution⁹.

We are investigating the possibility of a relationship between these grafted monolayers and Langmuir monolayers of long-chain amphiphiles at the air-water interface. In the latter case also, different states are observed for monolayer temperatures

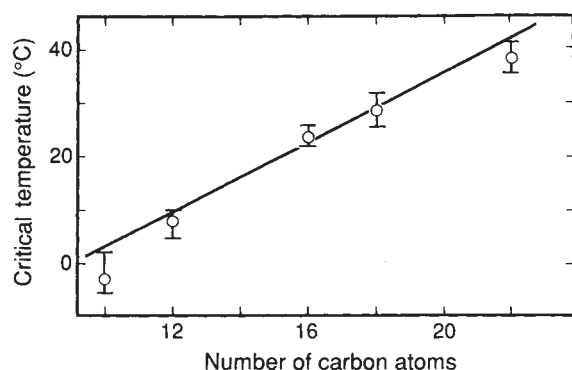


FIG. 4 Threshold temperature T_c for optimum grafting against the chain length n of the n -alkyltrichlorosilane. A linear variation of T_c is observed with a slope of $\sim 3.5^\circ\text{C}$ per additional methylene group.

above or below a characteristic temperature T_0 , the 'triple point'¹³. This temperature varies linearly with the alkyl chain length¹⁴, and it is striking that values published for fatty acids fall in the region as in the present experiments. If such an interpretation turns out to be correct, it would suggest that a lateral rearrangement occurs within the monolayer, following physisorption onto the substrate, and preceding chemical grafting. □

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The life span of the biosphere revisited

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A DECADE ago, Lovelock and Whitfield¹ raised the question of how much longer the biosphere can survive on Earth. They pointed out that, despite the current fossil-fuel induced increase in the atmospheric CO_2 concentration, the long-term trend should be in the opposite direction: as increased solar luminosity warms the Earth, silicate rocks should weather more readily, causing atmospheric CO_2 to decrease. In their model¹, atmospheric CO_2 falls below the critical level for C3 photosynthesis, 150 parts per million (p.p.m.), in only 100 Myr, and this is assumed to mark the demise of the biosphere as a whole. Here, we re-examine this problem using a more elaborate model that includes a more accurate treatment of the greenhouse effect of CO_2 (refs 2–4), a biologically mediated weathering parameterization, and the realization that

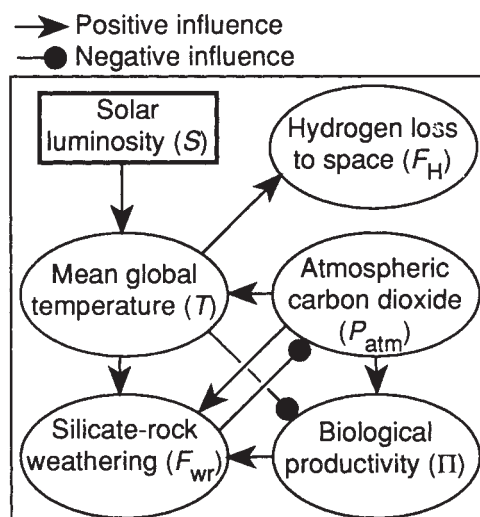


FIG. 1 Diagram illustrating the positive and negative influences represented in our model. System behaviour may be understood by examining the response to an increase in solar luminosity (S). Increasing S warms the Earth, increasing T . This enhances both the weatherability of silicate rocks (F_{wr}) and the rate of hydrogen escape to space (F_H). At temperatures approaching 50°C , temperature increases have a negative influence on biological productivity (Π). Enhanced silicate-rock weatherability draws down the atmospheric CO_2 concentration (P_{atm}). The lower CO_2 concentration tends to buffer T and F_{wr} and to reduce Π . Eventually, either lack of CO_2 , high temperatures, or the loss of water will limit the life span of the biosphere.

C4 photosynthesis can persist to much lower concentrations of atmospheric CO_2 (<10 p.p.m.)^{5,6}. We find that a C4-plant-based biosphere could survive for at least another 0.9 Gyr to 1.5 Gyr after the present time, depending respectively on whether CO_2 or temperature is the limiting factor. Within an additional 1 Gyr, Earth may lose its water to space, thereby following the path of its sister planet, Venus.

The problem of the life span of the biosphere has implications not only for the future of our planet, but also for the probability of finding biologically active planets in our galactic neighbourhood. As the Sun converts hydrogen to helium, its core becomes denser and hotter, increasing the rate of thermonuclear fusion; hence, standard solar models predict that the Sun has been getting more luminous with time^{7–9}. As solar luminosity increases, silicate rocks should weather more easily, thereby drawing down CO_2 from the Earth's atmosphere. This feedback mechanism should tend to buffer the Earth's temperature near its present value, both in the future and in the past¹⁰. Eventually, the CO_2 concentration may become so low that the megafloa existing at present will not be able to engage profitably in photosynthesis, effectively cutting off the carbon supply for the biosphere¹. Vanishingly low CO_2 concentrations would preclude further CO_2 -modulated thermal buffering. The Earth would then warm more rapidly, and much of the remaining biota might be pressed against thermal barriers to their survival. Ultimately, as the Sun continues to grow brighter, the Earth's surface water will be lost through photodissociation and escape of hydrogen to space. The loss of surface water would bring an indisputable end to the lifetime of the biosphere.

We developed a computer model (see Box 1 and Fig. 1) to analyse the situation described above. Because our model maximizes both the greenhouse effect and stratospheric water loss, and because we have estimated temperature and carbon requirements for the biosphere conservatively, our model should produce a minimum estimate for the biosphere's life span. We assume that future CO_2 emissions from volcanic and mid-ocean-ridge sources will continue at roughly the present rate, although there is some possibility that these rates will increase in the future¹¹. Thus, as long as most of the calcium and magnesium

BOX 1 Model description

Our computer model couples solar luminosity (S), the silicate-rock weathering rate (F_{wr}), and the global energy balance, to estimate the partial pressure of atmospheric carbon dioxide (P_{atm}), and biological productivity (Π) as a function of time (t) into the future.

The luminosity (S) of the Sun on the main sequence as measured at the Earth was calculated for this work by A. I. Boothroyd (personal communication) using a standard solar model⁹ with updated physical constants and ${}^7\text{Be}(e^-, \nu){}^7\text{Li}$ reaction rate. A curve fit to these model results yields

$$S(t) = (1 - 0.38t/\tau_0)^{-1} S_0 \quad (1)$$

with a mean error <0.6% for the interval $-4.5 \text{ Gyr} < t < 4.77 \text{ Gyr}$. Here t is time expressed as years from the present, $\tau_0 = 4.55 \text{ Gyr}$, and the subscript 0 refers to present-day values ($S_0 = 1.368 \text{ W m}^{-2}$). An energy balance may be written for the Earth by setting incoming shortwave radiation equal to the outgoing longwave radiation

$$(1 - a)S/4 = \sigma T_{\text{eff}}^4 \quad (2)$$

where a is the planetary albedo, σ is the Stefan-Boltzmann constant, and T_{eff} is the effective black-body radiation temperature of the Earth. T may be related to T_{eff} by the expression

$$T = T_{\text{eff}} + \Delta T \quad (3)$$

The greenhouse warming factor (ΔT) is a function of T (in K) and $\psi = \log P_{atm}$ (in bars):

$$\Delta T = 815.17 + (4.895 \times 10^7)T^{-2} - (3.9787 \times 10^5)T^{-1} - 6.7084\psi^{-2} + 73.221\psi^{-1} - 30.882T^{-1}\psi^{-1} \quad (4)$$

The planetary albedo (a) is a function of T

$$a = 1.4891 - 0.0065979T + (8.567 \times 10^{-6})T^2 \quad (5)$$

Equations (4) and (5) were developed using least-square fits to the results of 143 runs of a radiative-convective climate model²⁻⁴ with $0^\circ\text{C} \leq T \leq 100^\circ\text{C}$ and $10^{-8} \text{ bar} \leq P_{atm} \leq 10^{-2} \text{ bar}$; the mean errors were <0.5 K and <0.01 for equations (4) and (5), respectively. This radiative-convective climate model²⁻⁴ has an H_2O -saturated troposphere, producing maximum infrared trapping and maximum stratospheric water loss. Hence, this model should generate lower bounds on the potential life span of the biosphere. Because it is not known how clouds will respond to surface temperature changes, the effect of clouds on shortwave radiation forcing (14 W m^{-2})

and planetary albedo were held constant. We consider this to be a conservative assumption because, for large perturbations, clouds would probably act to stabilize Earth's temperature¹⁶.

The activity of H^+ in fresh soil water (a_{H^+}) can be calculated assuming equilibration of rain water with the soil CO_2 concentration (P_{soil}) and the atmospheric SO_2 concentration (0.2 p.p.b.). Equilibrium constants and relations for the carbon and sulphur systems may be found in refs 14 and 23, respectively. Experimental results in the acid pH range at 25°C with low CO_2 concentrations indicate that silicate dissolution rates are typically proportional to the hydrogen-ion activity to roughly the 0.5 power^{24,25}. Hence, incorporating the temperature dependency proposed by Walker *et al.*¹⁰, the silicate-rock weathering rate (F_{wr}), may be represented

$$\frac{F_{wr}}{F_{wr,0}} = \left(\frac{a_{\text{H}^+}}{a_{\text{H}^+,0}} \right)^{0.5} \exp \left(\frac{T - T_0}{13.7 \text{ K}} \right) \quad (6)$$

The exponent in equation (6) is in good agreement with the original formulation of Walker *et al.*¹⁰ (with an implied exponent of 0.6), developed using experimental results^{26,27} obtained at 100 to 200°C and 2 to 20 bars CO_2 .

Volk²⁸ parameterized P_{soil} as a function of Π and P_{atm}

$$\frac{P_{\text{soil}}}{P_{\text{soil},0}} = \frac{\Pi}{\Pi_0} \left(1 - \frac{P_{atm,0}}{P_{\text{soil},0}} \right) + \frac{P_{atm}}{P_{\text{soil},0}} \quad (7)$$

We extended Volk's²⁸ representation of Π by including a multiplicative, temperature-dependent term that produces maximum productivity when T is 25°C and zero productivity when T reaches 50°C

$$\frac{\Pi}{\Pi_{\text{max}}} = \left(1 - \left(\frac{T - 25^\circ\text{C}}{25^\circ\text{C}} \right)^2 \right) \left(\frac{P_{atm} - P_{\text{min}}}{P_{1/2} + (P_{atm} - P_{\text{min}})} \right) \quad (8)$$

A maximum possible biological productivity (Π_{max}) equal to twice the present productivity (Π_0) was deemed by Volk²⁸ to be the most defensible value, and is adopted for this study. The minimum CO_2 concentration under which C4 plants can grow (P_{min}) was conservatively estimated to be 10 p.p.m.. The value of $P_{1/2}$ is calculated by forcing equation (8) to yield $\Pi = \Pi_0$ when $T = T_0 (=15^\circ\text{C})$ and $P_{atm} = P_{atm,0} (=320 \text{ p.p.m.})$. Following Volk²⁸, we use $P_{\text{soil},0} = 10 P_{atm,0}$, as a representative soil $p\text{CO}_2(P_{\text{soil}})$.

The model is solved by setting $F_{wr} = F_{wr,0}$ until $P_{atm} = 1 \text{ p.p.m.}$; thereafter, P_{atm} is maintained at this value.

released in the weathering process accumulates in carbonate sediments, future weathering rates should be roughly the same as today's.

A principal difference between our model and that of Lovelock and Whitfield¹ is that our climate model is much more sensitive to CO_2 decreases below the present level of 350 p.p.m. Their proposed constant temperature relation between the atmospheric CO_2 partial pressure (P_{atm}) and solar luminosity (S) ($P_{atm}^{0.5} \propto 1.017 S_0 - S$) implies that the infrared trapping by 320 p.p.m. CO_2 is only $\sim 4 \text{ W m}^{-2}$. We calculate however, that the actual amount of infrared trapping by this concentration of CO_2 is 20.4 W m^{-2} , using a radiative-convective model²⁻⁴ at 15°C with relative humidity as specified by Manabe and Wetherald¹². This is about six times the radiative forcing (3.4 W m^{-2}) that would result from a CO_2 doubling in this model. (The absorption of infrared radiation increases roughly logarithmically with the CO_2 concentration because the strongest absorption bands, for example the $15\text{-}\mu\text{m}$ band, are already saturated near their centres.) If S increases with time according to equation (1) ($\sim 0.12 \text{ W m}^{-2} \text{ Myr}^{-1}$), then reductions in atmospheric CO_2 concentration could maintain a constant temperature for the next 0.9 Gyr. If we use the critical value for photosynthesis suggested by Lovelock and Whitfield ($P_{\text{min}} = 150 \text{ p.p.m.}$), our model calculates that 0.5 Gyr of the biosphere's life span remains, five times the Lovelock and Whitfield estimate. But, Lovelock and Whitfield ignored C4 plants, some of which can survive at CO_2 concentrations below 10 p.p.m. (refs 5 and 6). Using this criterion ($P_{\text{min}} = 10 \text{ p.p.m.}$), our model calculates that the remaining life span of the biosphere is 0.9 Gyr (Fig. 2).

There is reason to suspect that CO_2 starvation may not limit the life span of the biosphere. As solar luminosity continues to

increase, atmospheric CO_2 should continue to be drawn down; however, it may reach a minimum at which some C4 plants could survive. Equation (6) indicates that when the global mean temperature is above 300 K , silicate dissolution may proceed more rapidly than CO_2 and H_2SO_4 are supplied to the atmosphere by volcanism. This would leave an excess of Ca^{2+} and Mg^{2+} cations which could not be precipitated as carbonates or sulphates. They would probably precipitate in the oceans in a variety of silicate phases. Geological carbon inputs to the ocean and atmosphere must be largely balanced by carbonate sedimentation, suggesting that the oceans will continue to be saturated with respect to some carbonate phase. If future ocean waters are roughly in chemical equilibrium with silicates and carbonates, ocean pH would be buffered close to its present value¹³. If ocean pH increases by no more than half a pH unit and the oceanic Ca^{2+} concentration changes by no more than an order of magnitude, then application of carbonate equilibria¹⁴ indicates that atmospheric partial pressure of CO_2 ($p\text{CO}_2$) would be reduced no more than two orders of magnitude from today's value. Even if ocean chemistry is not this well buffered, Henry's law will partition more CO_2 into the atmosphere on a warmer Earth, so the atmosphere may still contain a few parts per million of CO_2 . Some present-day C4 plants could survive at these CO_2 concentrations^{5,6}, and aquatic microbial-based food chains could flourish at even lower CO_2 concentrations¹⁵.

If CO_2 starvation does not limit the life span of the nonmicrobial biosphere, high temperatures may. Even with negligible atmospheric CO_2 , global mean temperatures in our model exceed 50°C after $\sim 1.5 \text{ Gyr}$. Our model does not include the possible negative feedback of increasing cloud cover¹⁶; hence 1.5 Gyr should be considered a minimum time at which this

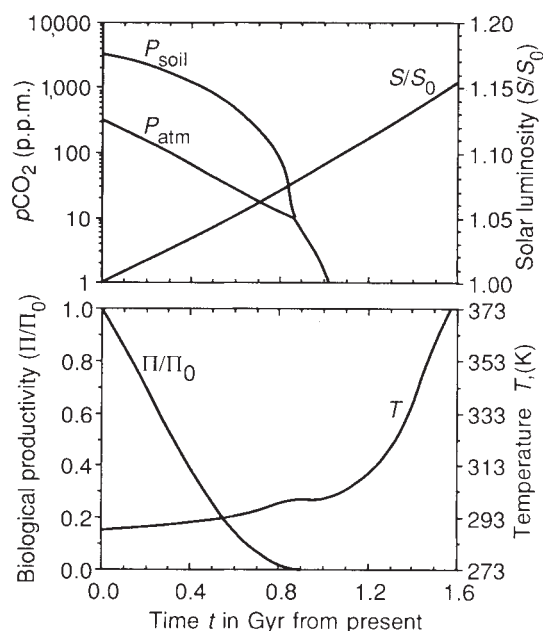


FIG. 2 Model results for the next 1.6 Gyr. The terrestrial biosphere, whose productivity (Π/Π_0) depends on an adequate supply of atmospheric CO_2 , maintains a gradient between soil and atmospheric CO_2 concentrations. For ~ 1 Gyr, the partial pressure of soil CO_2 (P_{soil}) and Earth's mean temperature (T) covary such that silicate-rock weathering consumes CO_2 at the modern rate. T is a function of both solar luminosity (S/S_0) and the atmospheric CO_2 concentration (P_{atm}). As S/S_0 increases, P_{atm} decreases, diminishing Π/Π_0 and with it the difference between P_{soil} and P_{atm} . When P_{atm} is too low for C4 plants to survive (~ 0.9 Gyr), then $P_{\text{soil}} = P_{\text{atm}}$. (In a C3-only world with $P_{\text{min}} = 150$ p.p.m., C3 plants survive ~ 0.5 Gyr.) After ~ 1 Gyr, silicate-rock dissolution exceeds volcanic CO_2 degassing; P_{atm} is then held at 1 p.p.m. The timescale for the loss of the oceans through hydrogen escape becomes limited by solar extreme ultraviolet when T climbs above 80°C (~ 1.5 Gyr). The loss of surface water by this process would take ~ 1 Gyr, and could ultimately limit the life span of the biosphere to ~ 2.5 Gyr.

temperature would be reached. Only prokaryotes and protozoa can live much above this temperature¹⁷. Beyond this time, planetary warming will probably accelerate. Atmospheric water vapour content increases exponentially with surface temperature if the relative humidity remains constant. This water vapour should trap outgoing longwave radiation and decrease the planetary albedo by absorbing solar near-infrared radiation. Both processes should contribute to planetary warming. In our model, the Earth's surface temperature increases from 50°C to 100°C in <0.2 Gyr. Some archaeobacteria might survive even higher temperatures^{18,19}, but all higher forms of life would certainly be eliminated by this stage.

The final sterilization of the Earth will occur when the planet loses its water. Today, the timescale for the loss of the oceans through photodissociation and hydrogen escape is much longer than the age of the Earth; as the atmosphere warms beyond 60 to 70°C , however, the H_2O mixing ratio in the stratosphere increases markedly²⁰. As surface temperatures approach $\sim 80^\circ\text{C}$, the stratospheric H_2O mixing ratio reaches $\sim 2.5\%$. Above this mixing ratio, the loss of hydrogen to space is limited by the solar extreme ultraviolet heating rate to $\sim 6 \times 10^{11}$ atoms $\text{H cm}^{-2} \text{ s}^{-1}$ (ref. 21), giving a timescale for ocean loss of ~ 1 Gyr. Hence, the complete elimination of water ~ 2.5 Gyr from now could bring terrestrial life to its final close. After Earth's water is lost, silicate-rock weathering will cease; hence, volcanic CO_2 should accumulate in the atmosphere, creating a climate much like that of Venus.

Events such as a collision with the giant comet Chiron²² or a radical reorganization of the carbonate-silicate cycle¹¹ could possibly bring the life of the biosphere to an earlier close. But given the otherwise conservative assumptions that we have made

in constructing our model, our results indicate that the biosphere will probably survive at least another 1 Gyr, and possibly much longer. This prediction ought to be encouraging to humans, as well, because it implies that we could survive for geologically long time periods if we can manage to cope with our other societal problems. □

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Electrical conductivity of carbon-bearing granulite at raised temperatures and pressures

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IT has long been recognized that the electrical conductivity of the lower continental crust is anomalously high. Both pore-saturating brines^{1–5} and conducting films of carbon at grain boundaries^{6–10} have been proposed to explain this, but the evidence remains inconclusive. Here we report measurements of electrical conductivity at high temperatures and pressures^{11–13} on samples of carbon-bearing and carbon-free granulites with a range of electrolyte saturations. The application of pressure to nominally dry carbon-free samples reduces the electrical conductivity as a result of a progressive reduction in pore connectivity, whereas the carbon-bearing samples show an increase in conductivity under the same conditions—an effect that we ascribe to reconnection of carbon conduction pathways during compaction. Moreover, we find a greater increase in conductivity with temperature for the carbon-bearing samples. In the light of work indicating that the abundance of carbon in high-grade rocks has been underestimated in the past^{7,8}, our results provide strong evidence for the role of carbon in lower-crustal conductivity.

Very few laboratory experiments have been done under fully simulated lower-crustal conditions to estimate the contribution of electrolytes to the *in situ* rock conductivity, and only one study, to our knowledge, examined carbon-bearing rock¹⁴. Here we examine carbon-bearing and carbon-free granulites under a

TABLE 1 Description of samples

Sample	Carbon content (wt%)	Orientation*	Saturation state	Maximum temperature (°C)	Maximum confining pressure (GPa)	Porosity (%)
Carbon-bearing granulites from Beni Bousera, northern Africa						
A1	2.70	Parallel	Saturated 0.5 M NaCl	700	0.2	0.88
A2	2.64	Perpendicular	Saturated 0.5 M NaCl	700	0.2	0.93
A3	2.71	Parallel	Unsaturated	25	0.4	0.91
A4	2.75	Perpendicular	Unsaturated	25	0.4	0.94
Mineral assemblage: clinopyroxene, orthopyroxene, plagioclase, garnet, graphite, biotite, spinels						
Carbon-free granulites from Ivrea Zone, northern Italy						
B1	<0.002	No foliation	Saturated 0.5 M NaCl	900	0.2	0.78
B2	<0.002	No foliation	Saturated distilled water	900	0.2	0.72
B3	graphite-free	No foliation	Unsaturated	25	0.4	1.03
B4	graphite-free	No foliation	Unsaturated	25	0.4	0.89
Mineral assemblage: clinopyroxene, orthopyroxene, plagioclase, spinels, hornblende						

Overview of the samples used in this work. Total carbon measurements were made using a Leco induction furnace and infra-red detector which was calibrated against samples of steel with accurately known carbon content. Thin-section studies of the granulites showed the absence of carbonate minerals. The total carbon content has, therefore, been taken as the carbon content and quoted as a percentage weight. The carbon content figures have an associated error of ± 0.1 in the range 1–10%. The carbon-free samples B1 and B2 had less than 20 p.p.m. carbon (the limit of instrumental resolution).

* Orientation of direction of electrical conductivity measurements with respect to the plane of foliation

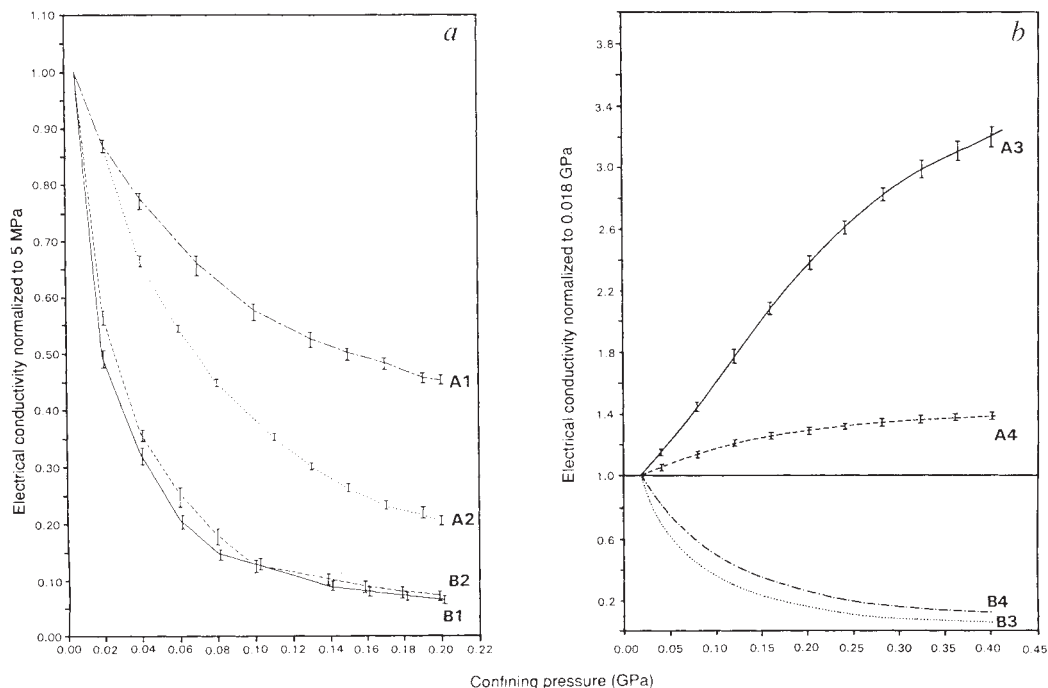
range of electrolyte saturations, confining pressures, pore pressures and temperatures to assess their contributions to the bulk conductivity of the rock and to help understand the nature of the rocks causing the high-conductivity zones in the lower crust.

When pressure is applied to an electrolyte-saturated rock, it reduces the rock's electrical conductivity. This is due to the progressive loss of porosity and pore connectivity through which the conduction occurs; the effect is smaller if there is significant conduction through the rock matrix^{2,3}. We found that the electrical conductivity of our electrolyte-saturated carbon-bearing granulite samples (A1, A2) decreased with the application of

confining pressures as expected. The decrease was, however, less than that for the carbon-free granulites, B1 and B2 (Fig. 1a). This is in itself unremarkable and can be explained if, of the two types, the carbon-free granulites have a higher porosity, a weaker matrix or a greater proportion of long thin cracks that tend to close on application of small confining pressures. In our work, however, the carbon-bearing granulite samples have a higher porosity (Table 1) and friable nature, and would be expected to lose much of their porosity, with associated loss of conductivity, when even small pressures are applied. Furthermore, the highly foliated nature of the carbon-bearing granulites suggests that much of their porosity is in the form of long thin

FIG. 1 a, Variation of the electrical conductivity of water- and brine-saturated carbon-free and carbon-bearing granulites as confining pressure is increased. Measurements were made with a Wayne Kerr B642 a.c. transformer bridge operating at 1,592 Hz. The samples were measured with a PTFE sleeve and two-electrode arrangement^{11–13} or with a metal sleeve and three-electrode guard-ring arrangement^{12,13}. All electrical conductivity measurements were taken after any transient changes in the pore structure had occurred. Error bars indicate the range of results from three experimental runs. A1 and A2: carbon-bearing, electrolyte-saturated, measurement direction parallel and perpendicular to planes of foliation respectively. B1

and B2: carbon-free, no foliation, saturated with electrolyte and distilled water respectively. The data are normalized to that at 5 MPa; absolute values for each of the samples at this pressure were as follows: A1, $5.01 \times 10^{-4} \text{ Sm}^{-1}$; A2, $2.51 \times 10^{-4} \text{ Sm}^{-1}$; B1, $1.995 \times 10^{-4} \text{ Sm}^{-1}$; B2, $2.24 \times 10^{-4} \text{ Sm}^{-1}$. b, Variation of the electrical conductivity of unsaturated samples of carbon-free and carbon-bearing granulites as confining pressure is



increased. Measurements and error bars as in a. A3 and A4: carbon-bearing, unsaturated, measurement direction parallel and perpendicular to planes of carbon foliation respectively. B3 and B4: carbon-free, unsaturated, no foliation. The data are normalized to that at 18 MPa; absolute values for each of the samples at this pressure were as follows: A3, $2.24 \times 10^{-5} \text{ Sm}^{-1}$; A4, $3.55 \times 10^{-6} \text{ Sm}^{-1}$; B3, $2.51 \times 10^{-6} \text{ Sm}^{-1}$; B4, $2.3 \times 10^{-6} \text{ Sm}^{-1}$.

cracks rather than less compressible, more rounded pores. If the pressure variation were solely due to electrolytic conduction through the pore fluid, one would expect that the flat thin cracks in the carbon-bearing granulites, confirmed by optical and electron microscopy, and deduced from mercury injection porosimetry, would make the electrical conductivity more sensitive to pressure than was observed. It seems, therefore, that sample pore-space or matrix properties cannot account for the different trends in electrical conductivity.

We expected the conductivity of nominally dry samples to mirror the behaviour of the fully saturated samples but to be lower overall. The reason for this was that the nominally dry samples were not oven-dried and would contain small amounts of remaining pore fluids. The carbon-free samples (B3, B4) showed lower conductivities than the saturated case, as expected. The conductivity of the unsaturated carbon-bearing samples (A3, A4) at near surface conditions was lower than that when saturated with electrolyte, as expected, but increased with the application of confining pressure (Fig. 1b). This behaviour is consistent with that of the saturated samples and indicates that

the lower rate of reduction of conductivity with pressure is not associated with electrolytic conduction but with some mechanism that becomes more efficient during compaction.

We found that the electrical conductivity of the electrolyte-saturated carbon-bearing samples increased more steeply with temperature than that for the electrolyte-saturated carbon-free samples (Fig. 2). This increased temperature dependence cannot be explained merely by additional carbon conduction, as graphite conduction is nearly independent of temperature. Further work on the carbon-electrolyte system is needed to ascertain whether the effect is connected with a carbon-water interaction or possibly the formation of carbon-sodium intercalation compounds at high temperature and pressure. These compounds have higher conductivity than graphite, but also have a negative coefficient of conductivity with temperature. The observed effect may be due to increased formation of such compounds as temperature increases.

Most measurements made in this work were at a fixed frequency of 1,592 Hz. The electrical conductivity of most rocks varies slightly with frequency, and considerable variations may occur in carbon-bearing rocks¹⁵. We measured the impedance spectra of rock samples A1, A2, B1 and B2 to check that the conductivities at the fixed frequency were representative. The

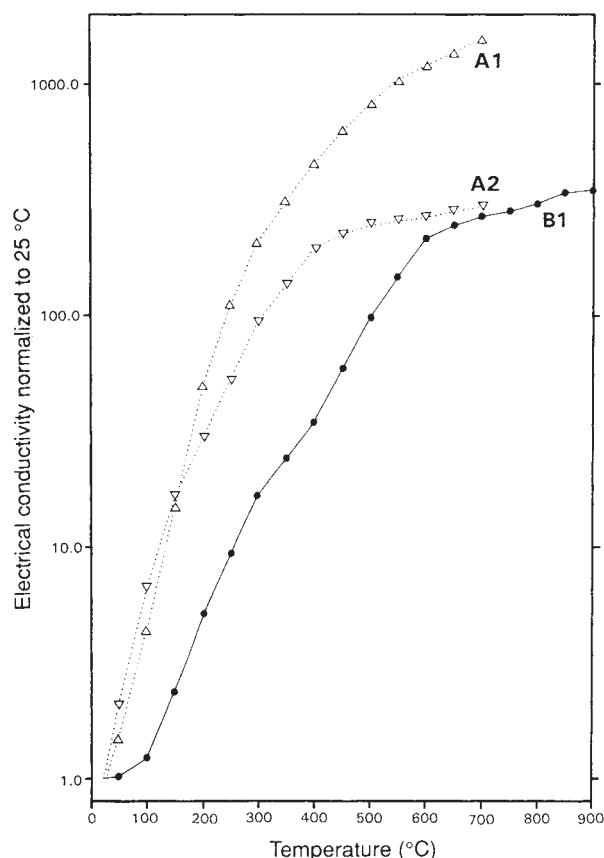


FIG. 2 Variation of the electrical conductivity of 0.5 M NaCl brine-saturated carbon-free and carbon-bearing granulites at constant confining pressure (0.2 GPa) and pore fluid pressure (0.18 GPa) at different temperatures (up to 800 °C). Measurements were made with a Wayne Kerr B642 a.c. transformer bridge operating at 1,592 Hz. The samples were measured with a metal sleeve and three-electrode guard-ring arrangement^{12,13}. The size of symbols indicates the approximate error associated with individual readings. A1 and A2: carbon-bearing, measurement direction parallel and perpendicular respectively to planes of carbon foliation. B1: carbon-free, no foliation. Conductivities during cooling coincided with those during heating. Heating/cooling hysteresis was observed on the first heating cycle. This was removed by holding the temperature high under pressure until all temperature-induced changes had occurred. The values given here are from subsequent heating and cooling cycles applied slowly. The results are normalized to that at 25 °C; absolute values for each of the samples at this temperature were as follows: A1, $1.26 \times 10^{-4} \text{ Sm}^{-1}$; A2, $0.631 \times 10^{-4} \text{ Sm}^{-1}$; B1, $1.26 \times 10^{-5} \text{ Sm}^{-1}$.

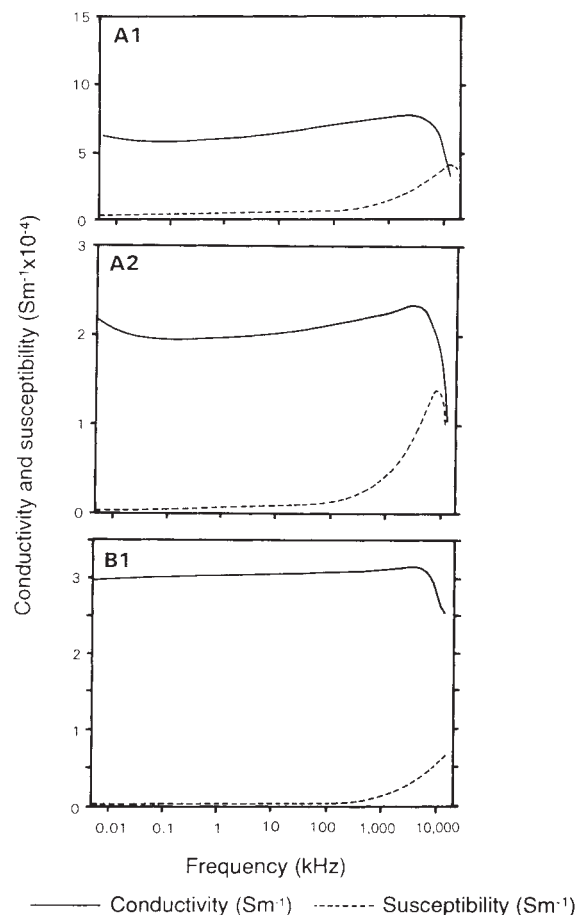


FIG. 3 The complex electrical conductivity of selected samples as a function of frequency of applied electric field. The measurements were made on a HP4192 LCR meter. Measurements of electrical conductivity and susceptibility were made at 20 frequencies per decade from 5 Hz to 20 MHz on samples saturated with 0.5 M aqueous NaCl solution with a two-electrode system. The electrodes were made from platinum-blackened platinum gauze to minimize electrode polarization at low frequencies. A1 and A2: carbon-bearing, electrolyte-saturated, measurement direction parallel and perpendicular to planes of foliation respectively. B1: carbon-free, no foliation, saturated with electrolyte.

mode of conduction in all samples is dominated by the in-phase conduction throughout the complete frequency range (Fig. 3). The electrical phase angles at 1,585 Hz, the nearest measured point to the frequency used in the main measurements, were -2.83° , -1.76° and -0.15° for A1, A2 and B1 respectively. This indicates that more out-of-phase conduction occurs in the carbon-bearing samples, although the effect is still small in comparison with the in-phase component. One possible explanation lies in the way in which charge carriers move between crystal lattice planes: the build up of charge carriers on alternate planes of a graphite lattice gives rise to local electrical fields that oppose the main field and generate the out-of-phase conduction. The presence of the out-of-phase conduction is therefore additional evidence that significant non-electrolyte-mediated conduction is occurring.

We propose that the increase in electrical conductivity in the unsaturated carbon-bearing samples is due to increased connectivity of carbon as the pore volume is lost and pore throats close down, leaving only compacted carbon conduction pathways. If so, the conductivity of the rock at high pressures would be expected to mirror that of carbon. Specifically, the rock's conductivity would depend only upon the amount and conductivity of the carbon fraction present, and have a negligible variation with pressure, as for pure carbon. We were able to create confining pressures of 0.4 GPa; to judge whether our hypothesis is correct, greater pressures are needed to compact the rock completely. Even at the highest pressures used here, the progressive loss of conduction by the small amounts of fluid still remaining in the pores unfortunately prevents quantitative comparisons with a dry rock containing only compact carbon and matrix minerals. It is clear, however, that some conducting mineral is responsible for the anomalous electrical behaviour. Quantitative mineralogical analysis of all the samples shows similar fractional amounts of all minerals but one. The exception is the presence of $\sim 2.7\%$ carbon in those labelled carbon-bearing¹².

Auger spectroscopy of igneous and metamorphic rocks has shown fine films of carbon covering grain boundaries^{7,8}. It has been postulated⁷ that decompression could irreversibly change the electrical conductivity of rocks by rupturing these films, and that laboratory measurements would not be able to duplicate the high conductivity in rocks even if the rocks were subjected to high pressures. Our experiment suggests that, at least to some extent, carbon grain-boundary films will reconnect under high pressures.

From the marked rise in the electrical conductivity of these saturated carbon-bearing samples as temperature and pressure increase, one would expect a high sample conductivity under lower-crustal conditions. The presence of carbon-bearing rocks similar to those studied here would raise the rock conductivity to moderate values if dry and to high values if also saturated with brines, producing values similar to those observed for the crustal high-conductivity zones by magnetotelluric methods¹⁶. It seems increasingly likely that plutonic and granulitic rocks with grain-boundary carbon could have electrical conductivities as high as those deduced in many areas of the lower continental crust. □

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A mantle metasomatic injection event linked to late Cretaceous kimberlite magmatism

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METASOMATISM of the lithospheric upper mantle by magmas and/or other fluids may induce significant changes in its chemical and mineralogical composition. It is not yet clear whether metasomatism takes place earlier than, concurrent with or subsequent to alkaline igneous activity and melt migration. Clues about the timing of metasomatism beneath the Kaapvaal craton in southern Africa are provided by metasomatized peridotites brought to the surface as xenoliths in young (80–95-Myr-old) group I kimberlites^{1,2}; the question that arises is whether the metasomatic alterations of the xenoliths are related to the host kimberlites themselves or to earlier igneous events such as the group II kimberlite eruptions (120–150 Myr)^{1,2}. Here we report a precise U–Pb age of 85 ± 2 Myr for zircons in a veined and metasomatized harzburgite xenolith from Kimberley, which indicates that this particular style of metasomatism (MARID-related³) is concurrent with the migration of the kimberlite magma that hosts the xenolith. This temporal link supports previous isotopic evidence⁴ for a genetic link between the group I kimberlite magmatism and the accompanying metasomatism.

Periodic volcanism has occurred on the Kaapvaal craton in southern Africa since the Archaean era, and each episode will have modified the composition of the subcontinental lithospheric mantle in which the magmas were generated or through which they passed, or in other words caused mantle metasomatism. The most recent volcanic rocks are the Cretaceous kimberlites which erupted in two pulses: group II (120–150 Myr) and

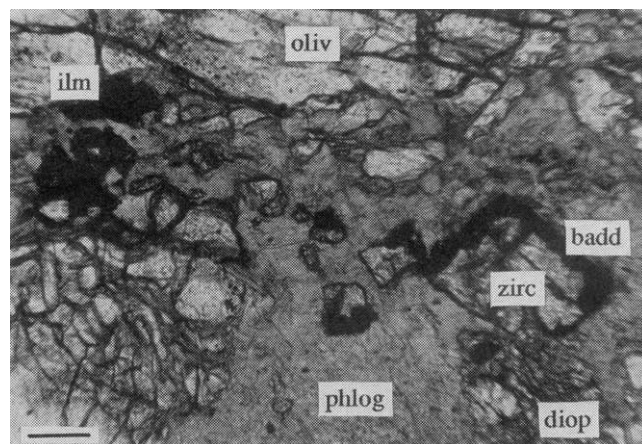


FIG. 1 Photomicrograph of veined harzburgite BD 3024 in plane polarized light, showing euhedral zircon crystals (zirc) with dark baddeleyite rims (badd), occupying a phlogopite-rich vein (phlog) cutting olivine (oliv). Additional phases: ilmenite (ilm); diopside (diop). Field of view $750 \mu\text{m} \times 525 \mu\text{m}$, scale bar $70 \mu\text{m}$.

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group I (80–95 Myr)^{1,2}. Group I, centred on the type-locality at Kimberley, has proved an important source for a wide variety of upper-mantle-derived, kimberlite-transported xenoliths ranging from peridotites and dunites, through pyroxenites and eclogites, to amphibole and phlogopite-bearing metasomites and discrete nodules (megacrysts)^{3,5,6}. Among these, Dawson and Smith³ recognized an igneous-textured paragenesis of the minerals mica–amphibole–rutile–ilmenite–diopside, having distinctive chemistry, and termed the MARID suite. Similar xenoliths were recognized elsewhere in southern African and Siberia^{3,7}. Dawson and Smith³ also recognized that some of the veined and pervasively metasomatized peridotite xenoliths from the Bultfontein Floors mine dumps at Kimberley have replacement mineral compositions that are broadly similar to those of the MARID suite (although the relationship between the two is not simple and some important chemical distinctions remain, possibly reflecting different degrees of interaction between veins and peridotite wall rocks^{5,8}). Composite nodules consisting of MARID nodules in direct contact with metasomatized peridotite are rare but do exist^{9–11}.

Zircon is rare in mantle rocks (all known occurrences in kimberlite are of xenocrystic or xenolithic origin¹²) but does occur in MARID xenoliths¹³. Here we report the dating of zircons from a metasomatic vein of MARID affiliation. Our interpretation of the data rests on the premise that the U–Pb isotope systems in these zircons record the actual time of their formation. Previously, the ages given by kimberlite-derived zircons (mostly megacrysts) have been assumed to be reset to the time of eruption and cooling^{14,15}. There is no accepted blocking temperature for Pb retention in zircon, however, nor any evidence that it lies below the equilibration temperatures of upper-mantle rocks. Indeed, there is evidence that old U–Pb ages are preserved in zircon megacrysts¹⁶ and in zircon inclusions in diamond¹⁷, both erupted in young kimberlites. On this basis, we reinterpret the young ages derived from most zircon megacrysts in kimberlites and related rocks as implying a close genetic relationship between the megacryst suites and their host eruptives.

The xenolith studied (BD 3024) is a veined and metasomatized 'fertile' spinel harzburgite, previously described and illustrated by Dawson¹⁰. It consists of a granular-textured palaeosome of partially serpentinized olivine, orthopyroxene and fingerprint Cr-spinel, cut by a series of subparallel veins up to 2 mm wide. These veins contain diopside and phlogopite, with minor disseminated ilmenite, zircon, calcite, apatite and serpentine. The wall-rock olivine at the vein margins is unaltered, but orthopyroxene is partially replaced by a fine symplectite of diopside plus phlogopite, and in some places by K-rich amphibole. Cr-spinel survives within the alteration zones. The vein assemblage shows chemical similarities with the MARID suite; specifically the alumina content of the ilmenite is low (<0.2 wt%) and, compared with typical primary-textured phlogopites from peridotite nodules in kimberlites, the vein micas are rich in Fe and Ti, and poor in Al and Cr^{3,18}. The diopside, too, is poorer in Al and Cr than most lherzolite diopsides, and the amphibole replacing orthopyroxene is potassic, sodic richterite.

The presence of zircon crystals up to 0.25 mm in size in the veins (Fig. 1) is reflected in the unusually high Zr content of the bulk rock (340 p.p.m.)¹⁰. The zircons are angular and have discontinuous rims of baddeleyite (ZrO₂), accompanied occasionally by zirconolite (CaZrTi₂O₇). The euhedral morphology of the zircons indicates that they crystallized with the primary vein assemblage and are neither inherited nor a secondary replacement phase. Three zircons were dated *in situ* using the SHRIMP ion microprobe at the Australian National University in Canberra¹⁹, after chips of the ANU zircon standard SL13 had been implanted into the analysed thin section. The results of five 25- μ m spot analyses are given in Table 1. Whereas most large mantle-derived zircons occurring in MARID, polymict

TABLE 1 SHRIMP U–Pb analyses of zircons in a metasomatic vein from Bultfontein

Spot	²⁰⁶ Pb*/ ²³⁸ U $\pm 2\sigma$	Age $\pm 2\sigma$	U	Th	Pb*	%c ²⁰⁶ Pb
1-1	0.01353 $\pm$ 68	87 $\pm$ 4	377	1,302	9	0.74
1-2	0.01357 $\pm$ 70	87 $\pm$ 4	323	1,192	8	1.09
2-1	0.01347 $\pm$ 68	86 $\pm$ 4	262	961	7	0.79
2-2	0.01314 $\pm$ 66	84 $\pm$ 4	377	1,173	9	0.94
3-1	0.01274 $\pm$ 66	82 $\pm$ 4	373	1,323	9	1.61
Average	0.01328 $\pm$ 30	85 $\pm$ 2				

Pb* refers to radiogenic Pb. U, Th and Pb concentrations are p.p.m. %c²⁰⁶Pb is the percentage of non-radiogenic ²⁰⁶Pb in the total. Spot notation is: grain number–site number.

and discrete nodules are characteristically low in U, Th and other trace elements^{12,14–16,20}, these small vein zircons are U-rich (average 340 p.p.m.) and particularly Th-rich (average 1,190 p.p.m.). Similarly high U contents (although with less Th) have been found in a small zircon inclusion in a Zaire diamond¹⁷. The high U and consequent high concentrations of radiogenic Pb in the vein zircons aided isotopic analysis with the ion probe, limiting the proportion of measured nonradiogenic ²⁰⁶Pb to less than 2% (Table 1). As the common Pb correction was based on the measured ²⁰⁷Pb (method described in ref. 21), only the ²⁰⁶Pb/²³⁸U age is quoted. All five analyses agree to within experimental error, yielding a mean age of 85 $\pm$ 2 Myr.

This result is indistinguishable from the 84 $\pm$ 1 Myr eruption age of the Bultfontein kimberlite at Kimberley, as given by Rb–Sr analyses⁴ of the phlogopites from MARID and related metasomatized nodules. It is also indistinguishable from an age of 82.7 $\pm$ 1.0 Myr (ref. 14) obtained from low-U anhedral zircons contained within the matrix of a Bultfontein polymict peridotite nodule (BD 2666)²², but is slightly younger than the 91.2 $\pm$ 1.4 Myr average age of loose zircon megacrysts¹⁴. Davis¹⁴ was not able to explain the discrepancy between the two zircon ages. P. J. Lawless (unpublished work) suggested that it might imply an extended period of volcanic activity, but if the low-temperature mica age is correct, the older age for the zircon megacrysts can be reinterpreted as the true mean age of their formation, close to but significantly before eruption. The overall age range for zircon megacrysts in the Kimberley pipes is 85–95 Myr. The broader inference is that the MARID rocks, related metasomites, megacrysts and polymict xenoliths found at Kimberley all formed in the upper mantle beneath the Kaapvaal craton over a short interval coincident with kimberlite generation and eruption. This is also in accord with the relatively unradiogenic Pb isotopic compositions of MARID K-richites from Bultfontein⁴, but is inconsistent with the Rb–Sr whole-rock ages of ~150 Myr reported for Bultfontein xenoliths^{23,24}. We consider the latter to be suspect in any case, as the data are too scattered to form true isochrons.

Taken a step further, these results lend strong support to the original interpretation of MARID xenoliths and associated metasomites as having been formed during injection and consolidation of kimberlitic melts. The model of Dawson and Smith³ involved cumulus crystallization at shallow depths in the mantle (within the known stability range of K-richite²⁵), from a magma of kimberlitic composition, accompanied by metasomatic alteration of the peridotitic wall-rocks caused by fluids migrating from the consolidating liquid. A second batch of ascending magma would then disrupt the MARID 'pegmatites' and the associated metasomatized wall-rocks, and carry them, together with other peridotite and eclogite fragments derived from greater depths, to higher crustal levels. Such a model contrasts with the suggestion⁹ that the bulk compositions of MARID xenoliths imply close genetic relations with lamproitic rocks. It must be remembered, however, that these xenoliths and veins do not represent frozen melt compositions, but rather

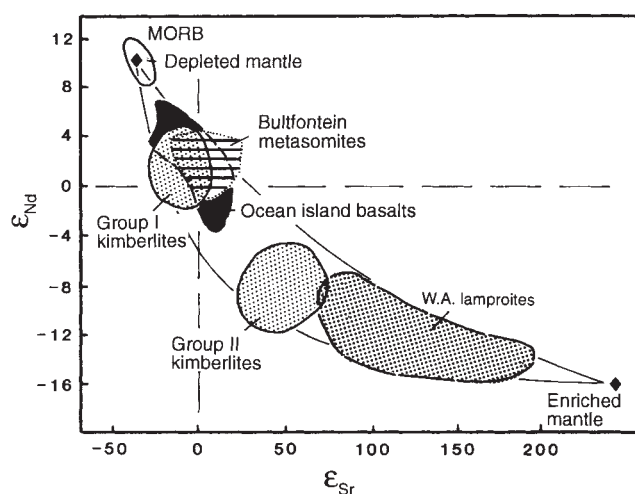


FIG. 2 Plot of initial Nd against initial Sr isotopic compositions for various mantle-derived rocks, showing fields for southern African Cretaceous kimberlites⁴, mineral phases in Bultfontein metasomites⁴, Western Australian Miocene lamproites²⁷, mid-ocean-ridge and ocean-island basalts. Also plotted are mixing lines between hypothetical enriched and depleted mantle endmembers, after McCulloch *et al.*²⁷.

the respective cumulate and infiltrate products of fractionally crystallized melts and/or associated fluids in an open system^{4,18}, so chemical resemblance may be fortuitous. Rather, the close similarity in age between the MARID-related metasomatism and the eruption of the Kimberley kimberlites supports the original idea that the MARID-forming fluid was a proto-kimberlitic melt. This is not to say that MARID-type metasomatism is a necessary precursor to all kimberlite genesis—the MARID suite is of restricted occurrence overall—but some form of metasomatism is an inevitable accompaniment. The minor age differences between zircons and other kimberlite minerals may result from the second magmatic pulse required to account for the disruption and deformation of MARID nodules before they are incorporated in the final erupted kimberlite.

Additional support for this model comes from the observations⁴ that the ranges in radiogenic isotopic compositions of the Bultfontein Floors metasomites overlap with those of the contemporaneous host group I kimberlites at Kimberley, but contrast strongly with the older, micaceous group II kimberlites of southern Africa (which are not known to contain zircons), implying geochemical differences between the mantle source rocks of the two groups^{1,2}. This point is illustrated by Fig. 2, adapted from Dawson²⁶, which also illustrates the distinct isotopic compositions of certain lamproites²⁷ (compare with ref. 9).

Although we have argued that the patent vein metasomatism observed in the Bultfontein peridotites represents a discrete, short-lived episode corresponding to the eruption of the Cretaceous group I kimberlites, we emphasize that alkaline volcanism on the Kaapvaal craton has occurred periodically since the Archaean era. Radiogenic studies indicate that there have been metasomatic enrichment events of comparable antiquity in the mantle. As the products of such events could be stored for long periods in the thick sub-cratonic lithospheric keel²⁸, there is ample scope for ancient relict-enriched mantle to be involved in younger igneous events. □

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Evidence from coupled ^{147}Sm – ^{143}Nd and ^{146}Sm – ^{142}Nd systematics for very early (4.5-Gyr) differentiation of the Earth's mantle

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THE volume of early Archaean crust that still survives today is very small (less than 1% of the present continental volume). This has been interpreted as indicating that crustal growth did not begin until about 4.0 Gyr ago, before which the silicate Earth remained well mixed and essentially undifferentiated. But the existence in some early Archaean rocks^{1–6} of inferred initial $^{143}\text{Nd}/^{144}\text{Nd}$ ratios higher than that of the bulk Earth suggests that by 3.8 Gyr ago the volume of the crust was as large as about 40% of the present value^{3,7,8}. Given the apparently low rate of recycling in the Hadean era (that is, before 4 Gyr ago)^{7,8}, consideration of ^{147}Sm – ^{143}Nd systematics then suggests that primordial differentiation of the Earth may have begun ~4.5 Gyr ago. Here we present evidence for early differentiation, based on measurements of $^{143}\text{Nd}/^{144}\text{Nd}$ and $^{142}\text{Nd}/^{144}\text{Nd}$ ratios in a ~3.8-Gyr-old supracrustal rock from Isua, West Greenland. Coupled $^{146,147}\text{Sm}$ – $^{142,143}\text{Nd}$ systematics suggest that fractionation of Sm/Nd took place 4.44–4.54 Gyr ago, owing to extraction of a light-rare-earth-element-enriched primordial crust.

^{146}Sm decays to ^{142}Nd with a half-life of 103 Myr. The presence of 'live' ^{146}Sm in the early Solar System is well established on the basis of several achondrite mineral isochrons; the initial abundance of ^{146}Sm is now established^{4,9–14} at $^{146}\text{Sm}/^{144}\text{Sm} = 0.0078 \pm 0.0010$ at 4,566 Myr. Because ^{146}Sm decays rapidly, becoming effectively extinct by 4.3 Gyr, it can be used to investigate differentiation processes active during the first 250 Myr of Earth history. This method has been successfully applied to lunar samples and SNC meteorites¹⁰, and the results constrain the time of early differentiation of the Moon and Mars.

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TABLE 1 Nd isotopic data

Sample	Method	Number of ratios	$^{144}\text{Sm}/^{144}\text{Nd} \times 10^6$	$^{142}\text{Ce}/^{142}\text{Nd} \times 10^6$	$^{142}\text{Nd}/^{144}\text{Nd}$	$100 \times \varepsilon^{142}\text{Nd}(0)$
Isua 1	A	200	14.7 ± 0.5	13.1 ± 0.4	1.1382858 ± 114	23 ± 10
Isua 2	A	120	<0.5	27.4 ± 0.3	1.1383063 ± 91	41 ± 8
Isua 3	A	470	<0.2	24.8 ± 0.3	1.1382972 ± 68	33 ± 6
Isua 4	B	940	<0.2	20.0 ± 0.3	1.1382972 ± 57	33 ± 5
Isua 5	C	840	<0.1	36.2 ± 0.4	1.1382945 ± 80	31 ± 8
Isua 6	C	1,000	<0.05	0.15 ± 0.05	1.1383111 ± 82	45 ± 8
Mix 1	C	720	<0.05	0.25 ± 0.14	1.1382893 ± 52	26 ± 7
Mix 2	C	1,200	<0.05	0.20 ± 0.13	1.1382822 ± 63	20 ± 7
BCR-1	C	2,000	<0.05	0.92 ± 0.12	1.1382657 ± 51	5.4 ± 6.2
Ndβ 1	A	500	<0.2	<0.4	1.1382584 ± 57	-1.1 ± 5.0
Ndβ 2	A	500	<0.2	<0.3	1.1382609 ± 55	1.1 ± 4.8
Ndβ 3	A	800	<0.2	<0.4	1.1382670 ± 52	6.5 ± 4.6
Ndβ 4	A	620	<0.2	<0.2	1.1382538 ± 73	-5.1 ± 6.4
Ndβ 5	A	280	<0.2	<0.4	1.1382510 ± 72	-7.6 ± 6.3
Ndβ 6	B	2,600	<0.2	<0.4	1.1382615 ± 28	1.7 ± 2.5
Ndβ 7	B	3,160	<0.2	<0.4	1.1382589 ± 28	-0.6 ± 2.5
Ndβ 8	B	2,260	<0.2	<0.4	1.1382582 ± 32	-1.2 ± 2.8
Ndβ 9	C	840	<0.05	0.13 ± 0.06	1.1382691 ± 74	8.4 ± 6.5
Ndβ 10	C	1,920	<0.05	0.00 ± 0.02	1.1382644 ± 35	4.2 ± 3.1
Ndβ 11	C	600	<0.05	0.05 ± 0.06	1.1382665 ± 101	6.1 ± 8.9
Ndβ 12	C	2,000	<0.05	0.20 ± 0.01	1.1382543 ± 46	-4.7 ± 4.0
Ndβ 13	C	760	<0.05	0.13 ± 0.01	1.1382505 ± 47	-8.0 ± 4.1
Ndβ 14	C	1,320	<0.05	0.18 ± 0.01	1.1382520 ± 48	-6.7 ± 4.2
Ndβ 15	C	1,400	<0.05	0.01 ± 0.03	1.1382501 ± 44	-8.4 ± 3.9
Ndβ 16	C	880	<0.05	0.01 ± 0.04	1.1382698 ± 117	9.0 ± 10.3
Ndβ 17	C	920	<0.05	0.06 ± 0.04	1.1382585 ± 88	-1.0 ± 7.7
Ndβ 18	C	1,760	<0.05	0.05 ± 0.03	1.1382607 ± 92	1.0 ± 8.1

Measured $^{142}\text{Nd}/^{144}\text{Nd}$ ratios were corrected for fractionation using $^{146}\text{Nd}/^{144}\text{Nd} = 0.724134$. The uncertainties in Nd isotopic ratios are 2σ of the mean of the interference-corrected block value distributions (internal errors). Uncertainties in the $\varepsilon^{142}\text{Nd}$ values reported for all sample runs have been expanded quadratically to include error in standard value determinations.

METHODS: A and B are alternative cup configurations used in static multicollection measurements; C is the configuration used in dynamic measurements. Isua runs 1–3 were made with cup configuration A with peak jump sequences to monitor ^{142}Ce and ^{144}Sm (run 1) and ^{142}Ce (runs 2 and 3). Isua run 4 (940 ratios) was made using a time-efficient 'fully static' cup configuration (B), which allowed direct monitoring of ^{140}Ce (and ^{147}Sm). Cup configuration A allowed simultaneous static collection of all seven Nd isotopes, but required peak jumps to monitor interfering elements. Configuration B was used to measure the masses 140, 142, 143, 144, 146 and 147 a.m.u. simultaneously, allowing fully static measurements of the most critical Nd isotopes and interfering element masses. Isua runs 5, 6, the mixtures and BCR-1 were done by dynamic multicollection using cup configuration C, which monitors mass 140 and generates multidynamic data for masses 142, 143, 144, 145 and 146 with fully exponential-law cup intercalibration and fractionation correction. In all cases, measured Nd isotopic ratios other than $^{142}\text{Nd}/^{144}\text{Nd}$ were found to be normal within uncertainty. $^{142}\text{Nd}/^{144}\text{Nd}$ values for the static measurements of the Ndβ standard (runs 1–8) and the Isua sample (runs 1–4) were obtained using the mean value of the dynamic measurements of the Ndβ standard (runs 9–18, 1.1382596) without propagation of its uncertainty. The numbers of ratios per run are listed for 8-s and 16-s beam integration intervals for static and dynamic measurements, respectively, at $^{142}\text{Nd}^+$ beam intensities of $\sim 7 \times 10^{-11}$ A for run durations of 6–60 hours. The ε and f values used in the text and this table are defined as $\varepsilon^{143}\text{Nd}(T) = \{[(^{143}\text{Nd}/^{144}\text{Nd})_{\text{sample}} / (^{143}\text{Nd}/^{144}\text{Nd})_{\text{CHUR}}^T] - 1\} \times 10^4$ where T is the age, and $(^{143}\text{Nd}/^{144}\text{Nd})_{\text{CHUR}}^T = (^{143}\text{Nd}/^{144}\text{Nd})_{\text{CHUR}}^0 - (^{147}\text{Sm}/^{144}\text{Nd})_{\text{CHUR}}^0 (e^{\lambda_{147}T} - 1)$. Present-day reference values for the bulk Earth are assumed to be equal to those in a chondritic uniform reservoir (CHUR), where $(^{143}\text{Nd}/^{144}\text{Nd})_{\text{CHUR}}^0 = 0.511847$, and $(^{147}\text{Sm}/^{144}\text{Nd})_{\text{CHUR}}^0 = 0.1967$ (ref. 4). $(^{143}\text{Nd}/^{144}\text{Nd})_{\text{sample}}$ is the ratio measured in the sample corrected for decay since the time of crystallization $T = 3.81$ Gyr. The $^{147}\text{Sm}/^{144}\text{Nd}$ fractionation factor relative to CHUR is given by $f_{\text{Sm}/\text{Nd}} = \{[(^{147}\text{Sm}/^{144}\text{Nd})_{\text{sample}} / (^{147}\text{Sm}/^{144}\text{Nd})_{\text{CHUR}}^0] - 1\}$. The $\varepsilon^{142}\text{Nd}$ values are defined as: $\varepsilon^{142}\text{Nd}(0) = \{[(^{142}\text{Nd}/^{144}\text{Nd})_{\text{sample}} / (^{142}\text{Nd}/^{144}\text{Nd})_{\text{CHUR}}^0] - 1\} \times 10^4$ where $(^{142}\text{Nd}/^{144}\text{Nd})_{\text{CHUR}}^0 = 1.1382596 (\pm 48, 2\sigma_m)$ follows from our working assumption of equality between CHUR and the present-day $^{142}\text{Nd}/^{144}\text{Nd}$ value in the CIT Ndβ standard obtained from our dynamic mode measurements.

The high initial $^{143}\text{Nd}/^{144}\text{Nd}$ ratios in the Isua (western Greenland) supracrustals³ and Saglek-Hebron (Labrador) gneisses¹⁵, which are ~ 3.8 Gyr old, show that the mantle reservoir from which these rocks were derived underwent fractionation and depletion hundreds of millions of years earlier than this. The $\varepsilon^{143}\text{Nd}$ value (for definition of ε and f -values see Table 1) in the mantle at $T_3 = 3.8$ Gyr can be modelled simply as a two-stage evolution from the origin of the Solar System at $T_1 = 4.566$ Gyr, with the first stage being that of an undifferentiated silicate Earth. The time of differentiation to form the depleted source region, T_2 , is obtained from

$$\varepsilon^{143}\text{Nd} \approx Q_{143} f_2^{\text{Sm}/\text{Nd}} (T_2 - T_3) \quad (1)$$

where $f_2^{\text{Sm}/\text{Nd}}$ is the Sm/Nd fractionation factor in the depleted mantle source, $Q_{143} \equiv 10^4 \lambda_{147} (^{147}\text{Sm}/^{143}\text{Nd})_{\text{CHUR}}^0 = 25.13 \text{ Gyr}^{-1}$ (ref. 4) and $\lambda_{147} = 0.00654 \text{ Gyr}^{-1}$ is the decay constant of ^{147}Sm . If instead a continuous or multistage process fractionated the Isua mantle source, then T_2 represents the mean age of the Sm/Nd fractionation in the source region¹⁶. T_2 is indeterminate in the ^{147}Sm – ^{143}Nd systematics because there are two unknowns (T_2 and $f_2^{\text{Sm}/\text{Nd}}$) and only one chronometric equation, so the

value of T_2 depends on how well $f_2^{\text{Sm}/\text{Nd}}$ can be estimated independently. If we apply a typical mid-ocean-ridge basalt (MORB) source value ($f_2^{\text{Sm}/\text{Nd}} \approx 0.2$)¹⁶ and $\varepsilon^{143}\text{Nd} = 3$ at 3.8 Gyr, then we obtain $T_2 \approx 4.4$ Gyr, suggesting that a very ancient epoch of differentiation was responsible for formation of the Isua mantle source.

Addition of the ^{146}Sm – ^{142}Nd chronometric equation in the coupled ^{146}Sm – ^{142}Nd systematics allows a unique solution for both the model age T_2 and the fractionation factor $f_2^{\text{Sm}/\text{Nd}}$ of the depletion in a two-stage differentiation model. Thus, by linking the extinct ^{146}Sm chronometer with the long-lived ^{147}Sm chronometer, we can precisely constrain the age of the initial differentiation in the mantle source region of the Earth's oldest surviving crustal rocks.

The $\varepsilon^{142}\text{Nd}$ evolution for a two-stage model is

$$\varepsilon^{142}\text{Nd} \approx Q_{142} f_2^{\text{Sm}/\text{Nd}} \left(\frac{^{146}\text{Sm}}{^{144}\text{Sm}} \right)_{T_1} \times [e^{-\lambda_{146}(T_1 - T_2)} - e^{-\lambda_{146}(T_1 - T_3)}] \quad (2)$$

where $\lambda_{146} = 6.73 \text{ Gyr}^{-1}$ and $Q_{142} \equiv 10^4 (^{144}\text{Sm}/^{142}\text{Nd})_{\text{CHUR}}^0 =$

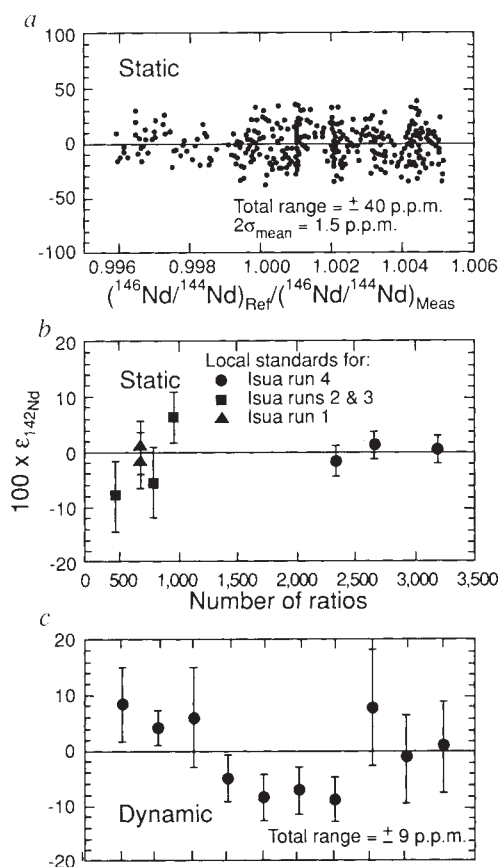


FIG. 1 a, Deviations ($100 \times \epsilon^{142}\text{Nd}(0)$, p.p.m.) of fractionation-corrected $^{142}\text{Nd}/^{144}\text{Nd}$ block data from the grand mean value for 410 blocks of data each with 20 integrations (8 s each) per block, against the fractionation factor obtained from $^{146}\text{Nd}/^{144}\text{Nd}$. Three filament loadings of 1 μg of the CIT Nd β standard were used to obtain this data base as the 'local standards' (standards run before and after each sample run) for Isua run 4. The total range in these blocks (± 40 p.p.m.) is only slightly greater than the magnitude of the mass-142 'anomaly' measured in the sample (Fig. 2). No fractionation trend is apparent in the data. Two sigma of the mean of this data is 1.5 p.p.m., such that with this technique it should be possible to resolve $^{142}\text{Nd}/^{144}\text{Nd}$ anomalies as small as 4 p.p.m. b, Deviations (p.p.m.) for our average values obtained from individual runs of the CIT Nd β standard plotted against the number of measured ratios for the static multicollection standards. The uncertainties are 2σ of the mean of the block values distribution (internal errors). c, Reproducibility for standards run in dynamic multicollection mode (plotted along the x-axis in run sequence 9–18). The uncertainties are again 2σ of the mean of the block values distribution (internal errors). For all runs, ion beam intensities for $^{142}\text{Nd}^+$ were $\sim 7 \times 10^{-11}$ A, with an amplifier noise level of $< 5 \times 10^{-17}$ A for a 64-s integration. Interfering ions were checked by scanning the mass region 130–155 with an ion counter. Only ^{142}Ce and ^{144}Sm were found to give direct interferences affecting our measurement of $^{142}\text{Nd}/^{144}\text{Nd}$. These interferences were always < 0.5 p.p.m. of the Nd isotope peaks for the Nd β standard.

354 (ref. 4). Equation (2) is exact for an 'initial' $\epsilon^{142}\text{Nd}$ value at $T_3 = 3.8$ Gyr. Identity with a present-day measured $\epsilon^{142}\text{Nd}$ value assumes that ^{146}Sm decay after T_3 contributes insignificantly to the final ^{142}Nd anomaly. This assumption is justified because *in situ* ^{146}Sm decay will not be significant for stage 3 (post-3.8 Gyr) residence in the crust at typical sample $f_3^{\text{Sm}/\text{Nd}}$ -values. Using $\epsilon^{143}\text{Nd} = 3$, $f_2^{\text{Sm}/\text{Nd}} = 0.2$ and $T_2 = 4.4$ Gyr in equation (2) yields $\epsilon^{142}\text{Nd} = 0.18$, whereas with $T_2 = 4.55$ Gyr we obtain $\epsilon^{142}\text{Nd} = 0.50$. As it is possible to measure $\epsilon^{142}\text{Nd}$ to better than 0.1 ϵ -units, as discussed below, the high initial $\epsilon^{143}\text{Nd}$ values found in many early crustal rocks imply that measurable $\epsilon^{142}\text{Nd}$ heterogeneities are probably present in many early Archaean rocks.

Our method uses a Finnigan MAT 262 mass spectrometer, with nine Faraday cups and an ion counter. Figure 1a demon-

strates that with careful work, this instrument should allow resolution of $^{142}\text{Nd}/^{144}\text{Nd}$ anomalies as small as 4 p.p.m. (0.04 ϵ -units).

We selected an Isua supracrustal sample (IE 715-28) with an apparent initial $\epsilon^{143}\text{Nd} = 4.0 \pm 0.5$ (ref. 3) at 3.81 Gyr (ref. 17) for precise determination of its $\epsilon^{142}\text{Nd}$ -value. Results for the CIT Nd β standard and repeat measurements of the sample and a 50:50 sample-standard mixture are given in Table 1 and shown in Fig. 2 along with an additional analysis of the ~ 14 -Myr-old Columbia River flood basalt standard BCR-1 ($\epsilon^{143}\text{Nd} \approx 0$). To ensure accuracy, measurements of $^{142}\text{Nd}/^{144}\text{Nd}$ in the Isua sample were made by three independent methods. Four static multicollection analyses of the sample were made using two different cup configurations: A (runs 1–3) and B (run 4). 'Local' standards (two or three runs made before and after each sample run) were used to determine the $^{142}\text{Nd}/^{144}\text{Nd}$ reference value because long-term reproducibility is degraded in the static method by long-term drifts in detector efficiencies. Reproducibilities of the local standards for sample runs 1–4 are shown in Fig. 1b. Agreement within internal (run statistics) 2σ errors is excellent for the run 1 and run 4 local standards. The lack of overlap of the mean for two out of the three local standards for runs 2 and 3, however, indicates that external (population statistics) errors exceed internal errors for this group. The very precise local standards for sample run 4 exhibit particularly good external agreement to within 3 p.p.m. total range. Two dynamic multicollection mass analyses of the sample (runs 5 and 6) and two of a 50:50 sample-standard (Isua plus Nd β) mixture were made using a third cup configuration, C. The dynamic multicollection method involves a trade-off in achievable precision to gain higher confidence of accuracy. Because detector efficiency drifts are corrected in the dynamic method, the long-term mean of standards was used to determine our best estimate of $^{142}\text{Nd}/^{144}\text{Nd}$ in the standard. Internal errors (2σ) for our 2,000 ratio dynamic standard runs (16-s beam integrations) are typically ± 6 p.p.m. (as compared to ± 3 p.p.m. for our $> 2,000$ ratio static standard runs with 8-s integrations), but the external error is more than twice as large ($2\sigma_p = \pm 13$ p.p.m.; Fig. 1c). Although this is not as good as the apparent precision achievable by the static multicollection method, it is good enough to resolve effects > 10 p.p.m. with multi-run analyses.

Interference levels were monitored by ion counting, sometimes with simultaneous Faraday cup monitoring. Isua runs 1–5 had small but significant corrections for interferences from ^{142}Ce (and in one case from ^{144}Sm). Isua run 6, mixes 1 and 2, and BCR-1 were done with very pure Nd samples with < 50 p.p.b. and < 1 p.p.m. interferences from ^{144}Sm and ^{142}Ce , respectively. In no case is the propagated uncertainty in an interference correction greater than 0.5 p.p.m. (2σ) in the $^{142}\text{Nd}/^{144}\text{Nd}$ ratio determination.

Figure 2 shows our measurements of $\epsilon^{142}\text{Nd}$ for the Isua sample. All six determinations show well-resolved positive anomalies. The average of the six sample measurements is 34 p.p.m. with an unweighted uncertainty of $2\sigma_m = \pm 7$ p.p.m. However, we believe the results of runs 3 and 4 to be far superior to the other Isua sample runs on the basis of our estimate of realistic analytical precision. By preferentially weighting these runs, we obtain a preferred value of 33 ± 4 p.p.m., $2\sigma_m$. Both mixtures also show resolved anomalies overlapping the expected value of 16.5 ± 2 p.p.m.. The single BCR-1 run agrees with the standard within its uncertainty.

The main part of Fig. 3 shows two curves for a two-stage differentiation model in the coupled $^{146,147}\text{Sm}$ – $^{142,143}\text{Nd}$ systematics. The insets show isotopic evolution curves in the ^{146}Sm – ^{142}Nd and ^{147}Sm – ^{143}Nd systems for two specific solutions (marked with black dots). The two curves correspond to $\epsilon^{143}\text{Nd} = +3.0$ and $+4.0$ in the mantle at 3.81 Gyr and represent the range of uncertainty in the initial $\epsilon^{143}\text{Nd}$ estimate for our sample at 3.8 Gyr. The measured value is 4.0 ± 0.5 , but other related samples from sequence B at Isua show a range of lesser values

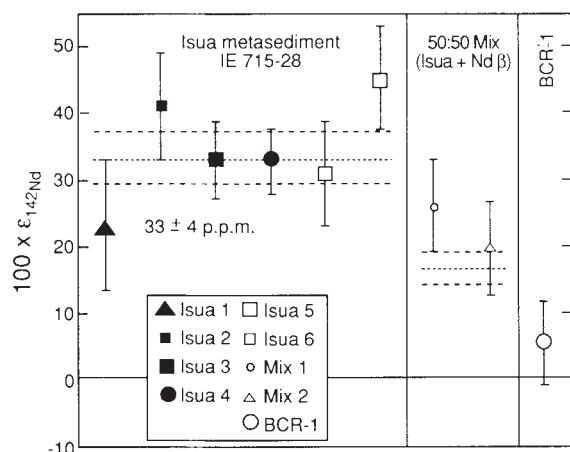


FIG. 2 Six independent multicollection measurements of the $^{142}\text{Nd}/^{144}\text{Nd}$ 'anomaly' in the Isua supracrustal sample 715-28, given as p.p.m. deviations ($100 \times \epsilon_{142\text{Nd}}(0)$) from the composition of the Nd β standard. As shown, there is reasonable agreement between all six measurements using the internal errors for each run. The error bars shown have been expanded quadratically to include the uncertainties in determinations of the standard mean values. The number of ratios in the measurement is given in Table 1. The best static measurements of the Isua sample (3 and 4) were made using different cup configurations but agree within 1 p.p.m. A preferred 'best estimate' value of $+33 \pm 4$ p.p.m., $2\sigma_m$, obtained from these measurements, is shown by the broken horizontal lines passing through the Isua sample data. The two dynamic runs of the Isua sample (5 and 6) confirm the static runs (1-4), as do the two runs of the 50:50 sample-standard mixture within their external errors. The broken horizontal lines show the expected range based upon our best estimate of the sample anomaly. One run of a pure Nd fraction separated from the Columbia river basalt standard BCR-1 ($\epsilon_{143\text{Nd}} \sim 0$) is in good agreement with the Nd β standard within uncertainty. The BCR-1 data point is consistent with our working assumption: $(^{142}\text{Nd}/^{144}\text{Nd})_{\text{Nd}\beta} = (^{142}\text{Nd}/^{144}\text{Nd})_{\text{CHUR}}$.

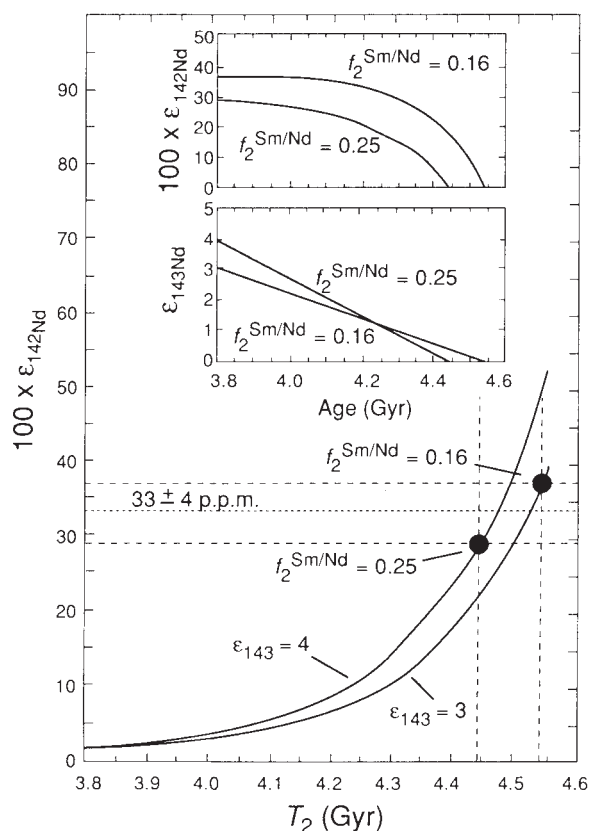


FIG. 3 Two loci of solution curves for a two-stage model in the ^{146}Sm - ^{143}Nd systematics are shown where the x-axis age is the formation time of the depleted reservoir, T_2 , and the y-axis is the magnitude of the ^{142}Nd 'anomaly' in p.p.m. ($100 \times \epsilon_{142\text{Nd}}$) relative to CHUR, assuming identical values of $^{142}\text{Nd}/^{144}\text{Nd}$ in Nd β and CHUR. The curves were drawn for initial $\epsilon_{143\text{Nd}}$ values of +4 and +3 at 3.8 Gyr, which are our estimated uncertainty boundaries for the Isua mantle source of our sample at that time. The constraint from our mass-142 anomaly measurement in Isua sample 715-28 (33 ± 4) is shown as a horizontal band. The intersection of this band with the solution curves for $\epsilon_{143\text{Nd}} = +3$ and +4 defines the vertical band intersecting the time axis. The resulting model age for the depleted mantle reservoir (and its enriched complement) is 4.44-4.54 Gyr. Shown in the inserts are conventional CHUR-normalized isotopic evolution plots for the ^{147}Sm - ^{143}Nd (lower inset) and ^{146}Sm - ^{142}Nd (upper inset) systematics. Isotopic evolution curves in these diagrams show the growth in $\epsilon_{143\text{Nd}}$ and $\epsilon_{142\text{Nd}}$ with time for specific solutions marked as solid dots on the main diagram. These points represent the maximal error boundaries for solutions of our ^{142}Nd anomaly measurement in the coupled Sm-Nd systematics for the range $+3.0 \leq \epsilon_{143\text{Nd}} \leq +4.0$. Each solution uniquely constrains both T_2 and $f_2^{\text{Sm/Nd}}$. Their respective f_2 -values are given in the main plot and are used as labels in both inserts. T_2 values are 4.44 Gyr and 4.55 Gyr for $f_2^{\text{Sm/Nd}} = 0.25$ and $f_2^{\text{Sm/Nd}} = 0.16$, respectively. This range of inferred $f_2^{\text{Sm/Nd}}$ -values is coincident with estimates of $f_2^{\text{Sm/Nd}}$ in the contemporary MORB source.

down to +3. The 33 ± 4 p.p.m. constraint from our mass-142 anomaly measurement is shown as a horizontal band. Intersection of this band with the two-stage growth model curves for $\epsilon_{143\text{Nd}} = 3$ and 4 defines the vertical band intersecting the x-axis which determines the age of differentiation (T_2) of the mantle source. The resulting model age for the depleted mantle reservoir (and its enriched crustal complement) is 4.44-4.54 Gyr. For so high an estimate of the reservoir age, continuous growth models closely approximate the two-stage model, making the estimate essentially independent of the model considered¹⁸.

The surfaces of the Moon, Mars and Mercury reveal crater-saturated regions of ~4.5-Gyr-old 'primordial crust'. The Earth's oldest known *in situ* crustal units (Isua¹⁷, Acasta¹⁹, Nulliak-Uivak¹⁵, Mt Sones²⁰, Anshan²¹) are all 3.8-4.0 Gyr old, leaving a 500-Myr gap in the crustal record. The only known material surviving from before 4.0 Gyr is a sedimentary component (containing datable detrital zircons) in Western Australia indicating the existence of apparently granitic continental crust at 4.2 Gyr

(refs 22-24). Both the mean and two-stage model ages of the light-rare-earth-element (LREE)-depleted Isua source reservoir are consistent with extraction of a complementary LREE-enriched 'primordial' crustal reservoir at ~4.5 Gyr, immediately following accretion and the creation of the Moon (probably) by a giant impact. This 'primordial' crust is analogous to the ancient cratered highland terrains surviving on the surfaces of the Moon, Mars and Mercury. No trace of primordial crust has been found on the Earth's surface, probably because it was destroyed by early impact and later erosion, and was recycled into the mantle by tectonic processes, either as sediments or as subducted plates. The preservation of the ^{142}Nd anomaly in the Isua source for ~0.7 Gyr, until 3.81 Gyr ago, suggests that the Hadean enriched crust was static, and that recycling (and presumably therefore contemporary-style plate tectonics) did not start before ~4 Gyr ago. Since then, tectonic processes have destroyed all but isotopic vestiges of these 'lost terrains' of the early Earth.

Two basic uncertainties affect our interpretation. First, not all Isua samples have high apparent initial $\epsilon^{143}\text{Nd}$ values. A range from about 0 to +3 is observed for other lithologies at Isua^{2,3}. As shown in Fig. 3, reservoir ages obtained for $\epsilon^{143}\text{Nd} < 3$ are limited by the age of the Solar System (~ 4.566 Gyr). Some of the variability in apparent initial $\epsilon^{143}\text{Nd}$ is likely to be due to metamorphic effects. Measurements of $\epsilon^{142}\text{Nd}$ on samples with a variety of apparent initial $\epsilon^{143}\text{Nd}$ values could resolve this issue. Second, $^{142}\text{Nd}/^{144}\text{Nd}$ in the Nd β standard may not be equal to the bulk Earth value. New high-precision measurements of $\epsilon^{142}\text{Nd}$ in meteorites and young terrestrial basalts are required if we are to resolve this issue. $\square$

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The earliest Acheulean from Konso-Gardula

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KONSO-GARDULA is a palaeoanthropological area discovered by the 1991 Palaeoanthropological Inventory of Ethiopia^{1–5} in the southern Main Ethiopian Rift. The Konso-Gardula sediments span the period about 1.3–1.9 million years ago. They contain rich Acheulean archaeological occurrences. Vertebrate fossils include early *Homo*.

Konso-Gardula (KGA) is on the western flank of the rift in the Segen River's headwaters north of Konso town (Fig. 1). Precambrian basement and >50 m of Plio-Pleistocene deposits are exposed across 12 × 5 km between 1,200 m and 1,400 m altitude. The local dip of Plio-Pleistocene strata approximates local

TABLE 1 Mean laser-fusion $^{40}\text{Ar}/^{39}\text{Ar}$ dates for Konso Tephra

Sample number	Grain size (mm)	Grains (n)	Ca/K (Ave)	Ca/K $\pm$	Age (Myr)	$\pm$ (Myr)
24-91	1.0	10	0.162	0.176	1.34	0.04
16-91	1.0	10	0.046	0.071	1.38	0.07
1A-91	1-2	16	0.041	0.061	1.37	0.03
1B-91	1.0	10	0.028	0.034	1.35	0.02
Chari Tuff*	1-1.5	8	0.047	0.018	1.34	0.02
6015-03	1-1.5	1	0.007		1.75	0.13
6015-06	1-1.5	1	0.009		1.48	0.01
Chari Tuff†	1.0	6	0.063	0.048	1.38	0.02
6018-02	1.0	1	0.421		1.52	0.02
6018-03	1.0	1	1.102		1.94	0.02
6018-07	1.0	1	0.130		1.62	0.01
6018-10	1.0	1	1.516		2.61	0.06
T-1	1.0	11	0.014	0.017	1.444	0.01
19-91	0.5	5	0.014	0.024	1.44	0.05
4A-91	1.0	8	0.114	0.078	1.66	0.03
4B-91	1.0	4	0.020	0.023	1.87	0.04
6-91	0.5	2	0.016	0.023	1.84	0.12
KBS Tuff‡	1.0	10	0.050	0.026	1.895	0.01

Grain size (mm) is given for the average maximum dimension of the feldspar population. n is the number of single-grain laser-fusion measurements. Ca/K, an average and standard deviation of n grains, is derived by multiplying the measured $^{37}\text{Ar}/^{39}\text{Ar}$ ratio of each grain by 1.96. The mean age and error (± 1 s.d.) are weighted mean values derived from the single-grain results, and are computed using an inverse variance weighting factor that uses deviations about a weighted mean to determine the weighted error^{29,30}. Individual grain errors (not shown) reflect errors in J (an irradiation parameter) and in the determination of Ar isotopic ratios, which in turn propagate errors in discrimination and errors in Ar beam intensities from sample and blank²⁹. Data in italics are excluded from the mean age. Samples of the Chari and KBS Tuffs were collected and provided by Garniss H. Curtis: Chari Tuff*, GKC-815; Chari Tuff†, GKC-818; and KBS Tuff‡, ER 131-0001. Clear, euhedral feldspars from the coarsest sieve fraction were hand-picked and irradiated for 0.5 h at 8 MW at the Omega West research reactor of the Los Alamos National Laboratory, which has a fast neutron fluence of $5.7 \times 10^{13} \text{ n cm}^{-2} \text{ s}^{-1}$. Cadmium shielding was used to reduce the thermal neutron production of ^{40}Ar . After irradiation, samples were transferred to a copper holder and loaded onto the extraction line for overnight bakeout at $\sim 200^\circ\text{C}$. Single grain samples are fused with an 8 W argon-ion laser. Abundances of the Ar isotopes are measured on a MAP-215 noble gas mass spectrometer fitted with a Balzers electron multiplier operating at a gain of about 20,000. The volume of a typical sanidine grain measured in this study is $\sim 1 \times 10^{-3} \text{ cm}^3$, corresponding to a sample mass of 3 mg per grain. Typical system blank volumes of ^{40}Ar , ^{39}Ar , ^{38}Ar , ^{37}Ar and ^{36}Ar are 4, 2, 0.08, 0.3, and $0.3 \times 10^{-17} \text{ mol}$, respectively. Neutron fluence monitor and constants as in ref. 6. Detailed results for individual grain analyses are available from R.C.W. by request.

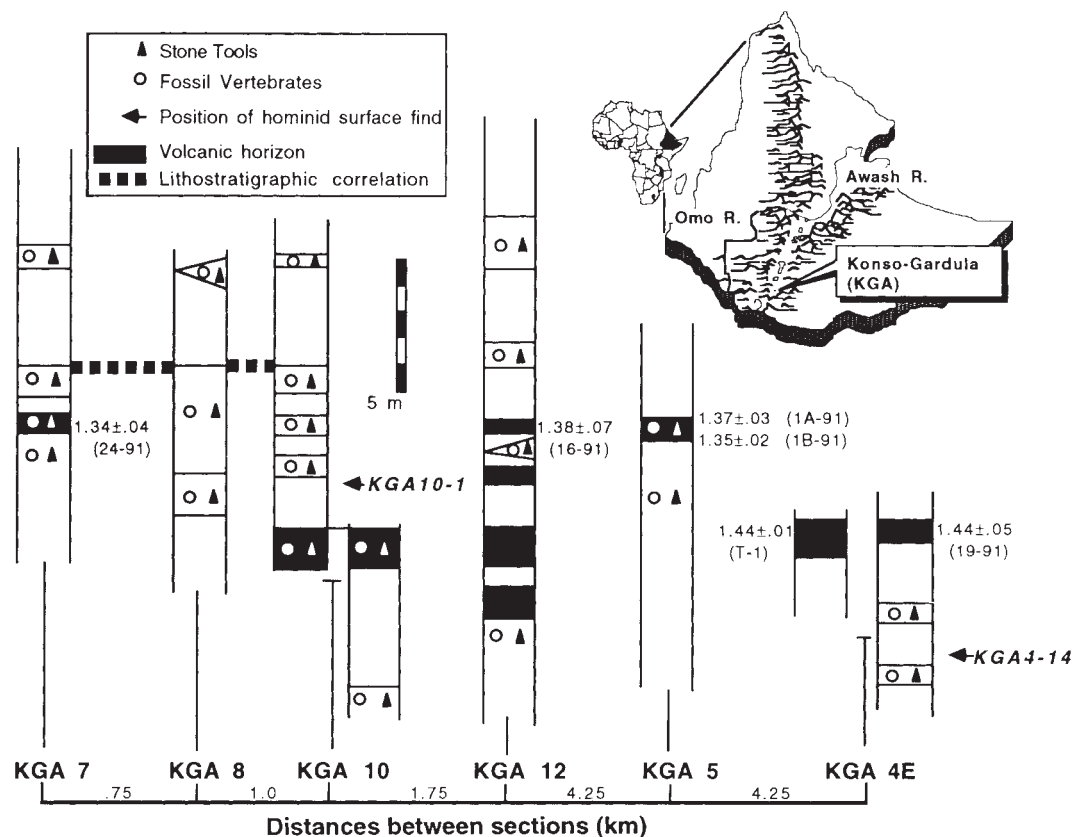
drainage, providing wide spatial exposure of these conglomerates, sands and gravels, silts, dark brown clays and interbedded volcanic tuffs.

Laser-fusion $^{40}\text{Ar}/^{39}\text{Ar}$ dating studies^{6,7} were done on KGA and Turkana Basin⁸ rocks (Table 1). Analyses of these tephra yield dates of 1.34, 1.38 and 1.36 million years (Myr), respectively, and two samples of the Chari Tuff yield dates of 1.34 and 1.38 Myr. These results suggest that 1.36 ± 0.02 Myr is an accurate estimate of the Chari Tuff's age and that these Konso tuffs are temporally equivalent with the Chari Tuff at Koobi Fora.

In locality KGA 4E, tephra 19-91 is 3–5 m above a hominid molar, and is dated to 1.44 ± 0.05 Myr. An equivalent unit (Tuff T-1) located ~ 500 m north is dated to 1.44 ± 0.01 Myr. Tuffs in fault-bounded outcrops sampled near the western fault contact of the Konso beds date to 1.66 ± 0.03 (4A-91) and 1.87 ± 0.04 (4B-91) Myr. The oldest tephra at Konso are thus within errors of the KBS Tuff, which is dated here to 1.895 ± 0.011 Myr (Table 1).

The Acheulean was unknown in southern Ethiopia before the discovery of KGA. Stone tools are abundant and diverse, par-

FIG. 1 Stratigraphic and geometric relationships between the dated volcanic horizons, fossil vertebrate remains, and *in situ* archaeological occurrences at the main KGA localities. Transects across outcrops of KGA sediments intercepted archaeological and faunal remains. Localities and sample locations were mapped on aerial photos and by GPS placement. Hominid surface finds were assessed in the field to have derived from the local outcrops depicted here (based on fragment scatter and preservation style).



ticularly in strata immediately above and below the tuff dated to 1.34–1.38 Myr. Archaeological and palaeontological remains are concentrated in thin (10 cm) sand lenses to thicker sand and silt strata (Fig. 1). Many vertical outcrops reveal artefacts and fossils *in situ*, often densely concentrated. Evidence of fluvial transport common in other Acheulean assemblages^{9–11} is absent. Tools were made from basalt, quartz, quartzite and silicic volcanic rock.

The pooled tool assemblage from KGA 3, 5 and 7–12 is dominated by roughly made bifaces and trihedral picks from cobbles, blocks, cores and flakes. Bifaces often display cortical butt and deep oblique flake scars. Some bifaces and picks attain great length (268 × 240 × 70 mm handaxe; 270 × 75 × 67 pick), but basic morphology of type tools is constant over a wide size range. Flakes are generally untrimmed, with a virtual absence of typologically diversified, retouched tools. Cleavers are rare and spheroids absent. Typologically, these assemblages best match those from middle Bed II at Olduvai (EF-HR, SHK; ref. 9).

The KGA artefacts are closely associated to fossilized bones. Evidence of hominid-induced modification of large mammal bones includes percussion pits and striae, internally conchoidal flake scars and cutmarks. Palaeontological resources are rich; fossils were observed in all KGA localities. Excellent bone preservation will enhance archaeological investigation of *in situ* assemblages. Biochronological placement of the vertebrate fauna (Table 2) is consistent with the radiometric dates.

A hominid upper third molar and a nearly complete left mandible with condyle and P₄–M₃ were recovered from KGA. Two¹², and possibly three^{13,14} early hominid species are recorded in contemporary rocks of the Turkana Basin and Olduvai: *Australopithecus boisei* and *Homo erectus*. Both 1991 KGA hominid specimens are attributed to *Homo* because they lack specialized characters¹³ of robust *Australopithecus*. The mandible is morphologically and metrically similar to *Homo erectus* specimens (for some, *Homo ergaster*¹³), particularly KNM-ER

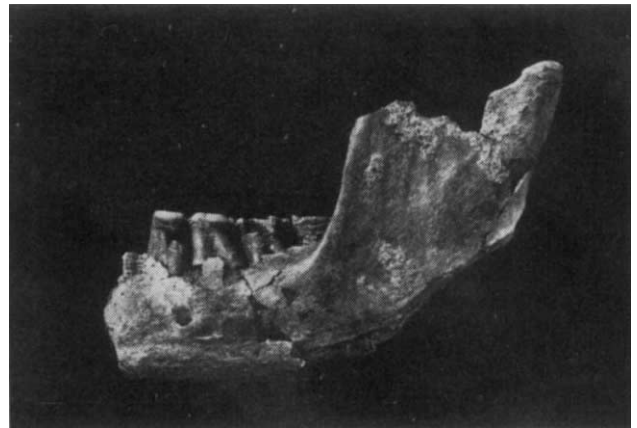
992. As in other early African *H. erectus*, the KGA 10-1 mandible has a very robust corpus (Fig. 2). The M₃ is distinctly smaller than M₁ in the M₂ > M₁ > M₃ size sequence. The M₁ shape index does not exhibit the buccolingual narrowing of *H. habilis* counterparts. The M₃ wear pattern has an exaggerated distolingual component, as in KNM-ER 730. Molars show a low level of root bifurcation as in KNM-ER 992. Interproximal grooves between all mandibular teeth probably indicate modification by toothpick¹⁵ or sinew¹⁶ manipulation. This is among the largest of the still limited sample of early African *H. erectus* mandibles¹³. It is too large for a cranium the size of KNM-ER 3733 and could be a better size match for the OH 9 cranium.

Biological and technological dynamics involved in the origin of the Acheulean and the origin and early evolution of *H. erectus* are poorly understood. Olduvai and west Natron localities were previously considered the earliest evidence of the Acheulean. Because only Bed I has yielded reliable radiometric dates⁶, the ages of these early Acheulean sites were poorly constrained. The KGA results supplement precise Ar/Ar dating results at Olorgesailie^{17,18} and Kesem-Kebena⁴ to demonstrate great antiquity for the Acheulean. It appears abruptly in eastern Africa at 1.4 Myr, following a million years of exclusively Oldowan occurrences¹⁹. Compared to these, the Acheulean's earliest manifestation at KGA shows surprising control over raw material and tool form. The temporal stability of the Acheulean industrial complex is thereby emphasized. Also surprising is the spectacular abundance of KGA artefacts and the apparently intensive Lower Pleistocene occupation of this basin. Contemporary deposits, in the nearby Turkana Basin (>200 km away) yielded many hominid fossils, but definitive Acheulean assemblages are lacking at Koobi Fora²⁰. This difference may relate to the close proximity of raw material sources to the KGA discard loci.

Leakey attributed the origin of the Acheulean at Olduvai to immigrant *Homo erectus*⁹. Subsequent discovery of terminal

TABLE 2 Faunal list for selected Konso-Gardula localities

Taxa	KGA locality											
	3	4E	4W	5	6	7	8	10	11	12		
Elephantidae												
<i>Elephas recki recki</i>												
<i>Elephas recki recki/ileretensis</i>	•	•	•	•				•	•	•	•	
Rhinocerotidae												
<i>Ceratotherium simum</i>	•			•							•	
Equidae												
<i>Hipparion libycum</i>		•									•	
<i>Equus cf. oldowayensis</i>	•	•	•	•			•	•	•		•	
Hippopotamidae												
<i>Hippopotamus/Hexaprotodon</i> sp.	•	•	•	•	•	•	•	•	•	•	•	
Suidae												
<i>Kolpochoerus majus</i>		•	•	•							•	
<i>Kolpochoerus olduvaiensis</i>			•						•			
<i>Metridiochoerus modestus</i>	•						•				•	
<i>Metridiochoerus hopwoodi</i>							•	•	•		•	
<i>Metridiochoerus compactus</i>									•			
Giraffidae												
<i>Sivatherium</i> sp.		•		•								
<i>Giraffa</i> sp.			•				•					
Bovidae												
<i>Tragelaphus cf. scriptus</i>									•			
<i>Tragelaphus strepsiceros</i>									•			
<i>Reduncini</i>	•	•	•	•	•	•	•	•	•		•	
<i>Kobus</i> sp.									•			
<i>Kobus cf. sigmoidalis</i>			•									
<i>Bovini</i>	•	•	•	•			•	•	•	•		
<i>Hippotragus</i> sp.									•			
<i>Damaliscus</i> sp.			•									
<i>Damaliscus niro</i>								•	•			
<i>Parmularius angusticornis</i>									•			
<i>Connochaetes</i> sp.									•			
<i>Alcelaphini</i>	•	•	•	•			•	•	•	•	•	
<i>Antilopini</i>		•		•								
<i>Gazella</i>							•			•		
<i>Aepyceros</i>			•						•			
Primates												
<i>Papio</i> sp.									•			
<i>Theropithecus oswaldi</i>			•									
<i>Homo</i> sp.		•										
<i>Homo erectus</i>									•			
Carnivora												
<i>Dinofelis</i> sp. aff. <i>piveteaui</i>		•							•			
<i>Hyaenidae</i>		•							•			
Reptilia												
<i>Python</i>											•	
<i>Crocodylus</i> sp.	•	•		•	•	•	•					
<i>Chelonia</i>	•	•				•	•					
Aves		•										
Pisces	•											

FIG. 2 Lateral view of the KGA 10-1 hominid mandible, attributed to *Homo erectus*.

evidence of occupation is unknown before ~1.0–1.4 Myr. The preponderance of the evidence is that *H. erectus* made its evolutionary appearance in Africa sometime before 1.7 Myr and dispersed into Eurasia within the next 0.3–0.5 Myr, at some point carrying the Acheulean. But the evidence is largely African, and the scope of appropriate deposits in intensively studied areas of southern Eurasia is limited. This combines with the lack of palaeoanthropological exploration in large parts of southern Eurasia to preclude definitive conclusions about the timing and direction of early hominid movements.

The last decade witnessed a proliferation of attempts to causally link hominid evolutionary events to changes in global climate^{23–25} (but for cautionary views, see refs 26, 27). Vrba²⁴ suggests that "... changes to open and arid conditions may have triggered the origin of *H. erectus* and of his characteristic tool kit ...", and that "the period around 0.9 Myr may also coincide with the earliest massive geographic expansion of any hominid species, namely *H. erectus*" (p. 421). The first African records of this taxon and the Acheulean, however, are now set solidly at 1.7 and 1.4 Myr, respectively. These appearances substantially postdate the global cooling between 2.4 and 2.8 Myr (ref. 28). The probable dispersal of *H. erectus* and the Acheulean from Africa into Eurasia substantially predates pronounced changes in global ice budget between 0.9 and 0.7 Myr. The period between 1.8 and 1.4 Myr was unmarked by major global climatic changes of comparable degree, but witnessed profound changes in hominid anatomy and technology. Research at early African occurrences like Konso-Gardula is needed to understand these changes. □

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Pliocene (1.7 Myr) *H. erectus*²¹ broadens its temporal and anatomical ranges (prompting some to recognize a new species for these early African remains, *H. ergaster*)^{13,14}. Discoveries at Olduvai and Turkana demonstrate close temporal proximity of *H. habilis* and early African *H. erectus*, but shed little light on the evolutionary relationships (punctuational or cladistic; *in situ* or allopatric) between the still inadequately sampled populations.

H. erectus was contemporary with the Acheulean at KGA and continued to be associated with it long after the subsequent extinction of other hominid lineage(s). The recent discovery of a possibly early Pleistocene hominid mandible at Dmanisi²² is a reminder of the still inadequate Eurasian early hominid record. Despite repeated claims to the contrary, well dated Eurasian

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Mitotic domains in the early embryo of the zebrafish

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At the midblastula transition in the zebrafish, three, and only three, spatially separate mitotic domains arise with distinctive cycle lengths and rhythms. As in *Drosophila*¹ and at about the equivalent stage, the mitotic domains reflect the fate map, but they do so only very crudely: two are extraembryonic and the third forms the entire embryo. The domains appear not to subdivide during gastrulation, when the germ layers form and when cells probably commit to their eventual fates. The domains may signal specification of morphogenesis rather than cell fate, because, shortly after they appear, each assumes a different role during epiboly, the first morphogenetic movement of the embryo. During meroblastic cleavage, and continuing in the early blastula, zebrafish blastomeres divide rapidly and synchronously. At the time of the tenth cleavage, the beginning of the midblastula transition, the cell cycle lengthens, and, as in *Xenopus*² and *Drosophila*³, cycle length comes under nucleocytoplasmic control (D.A.K. and C.B.K., manuscript in preparation). This nucleocytoplasmic control seems to be maintained during cycle lengthening in the next 2 or 3 cycles, comprising a midblastula transition period. We now show that functionally distinct subsets of cells that arise during this period have reproducibly different mitotic cycle lengths.

There are three of these domains, and they can be recognized morphologically (Fig. 1a and b). Two are of blastoderm cells, an outer enveloping monolayered epithelial layer (EVL), and an inner, or 'deep' mass of spherical cells. The third is the single giant multinucleate yolk cell; its syncytium of non-yolk cytoplasm and nuclei, the yolk syncytial layer (YSL), immediately underlies the marginal rim of the blastoderm^{4,5}. Video time-lapse analysis reveals that, during the midblastula transition (MBT) period, average mitotic cycle lengths within the domains diverge (Fig. 1c), YSL nuclei dividing most rapidly, and EVL cells most slowly.

Cell-lineage analysis (Fig. 2) readily distinguishes the domains, not only by mitotic cycle length, but also according to division synchrony. Examining single lineages that split into

separate domains sensitively demonstrates the origins of the cycle-length differences, indicated as 'mitotic borders' in Fig. 2c and d. Cycle lengths diverge rapidly upon the lineage segregation; for example in Fig. 2c, YSL nuclei divide more rapidly than their deep cell cousins as soon as the YSL forms at cycle 11. Similarly, in Fig. 2d, segregation of sister cells into the EVL and deep domains occurs after cycles 9 and 11, and the deep cells immediately divide more rapidly than their EVL sisters.

During the MBT period there is a close correlation between cycle lengths of daughter cell pairs with that of their mother (Fig. 3a–c). This is expected if control is by the nucleocytoplasmic ratio, for, if one assumes no growth during these divisions, the total volume of the daughters will equal that of the mothers. Then, beginning with daughters born in the late blastula, 4 h postfertilization, the mother–daughter correlation weakens, suggesting an end to the MBT period. In the deep domain, the cell cycle of the daughters becomes shorter than expected in late cycle 13 and cycle 14 (plateau in Fig. 3a). Afterwards, as deep

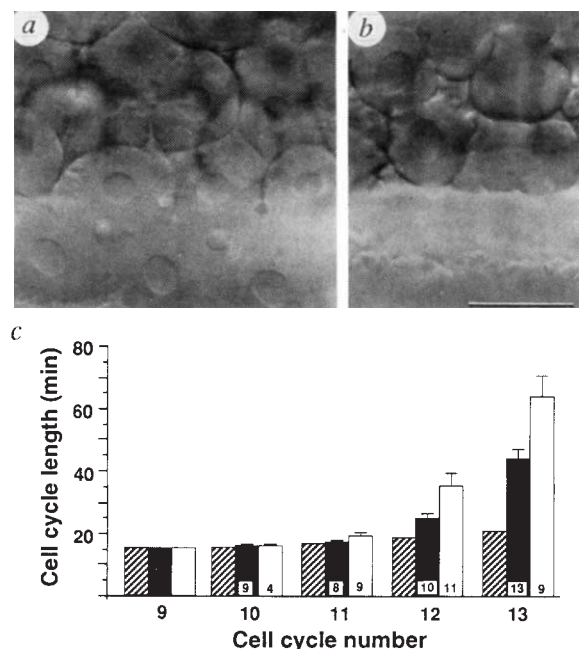


FIG. 1 Mitotic domains are evident during zebrafish MBT, from cycle 10 to 12. Note that cycle-10 corresponds to the 512-cell stage of ref. 10. a, Photomicrograph of the enveloping layer (EVL) of the blastoderm (cells in top half of photograph) and yolk syncytial layer (YSL) of the yolk cell (nuclei at bottom) at cycle 12. b, Photomicrograph of the deep cells (cells in top half of photograph) and yolk cell (bottom) at cycle 12, in a focal plane 20 μ m beneath a. Note spherical shape of the deep cells compared to the epithelial appearance of the overlying EVL cells. Scale bar for a and b, 30 μ m. c, Average cycle length of the mitotic domains, comparing the three mitotic domains of one embryo. Cycle 9 is typically the last synchronous short cycle (D.A.K. and C.B.K., submitted). Although the magnitude and initiation of the lengthening varied between embryos, all embryos had statistically significant differences ($P < 0.05$) between domains by cycle 12. Symbols: diagonal pattern, YSL; solid bars, deep cells; clear bars, EVL. Numbers in the bars (except for the YSL, which is part of a single cell) indicate the number of cells measured; error bars indicate the s.e.m.

METHODS. Eggs were collected from spawning zebrafish within 1.5 h of the time of fertilization, dechorionated, and monitored to verify the early staging of the eggs. Lineage analysis was done according to ref. 21 using 5 mg ml⁻¹ tetramethylrhodamine-isothiocyanate dextran (Molecular Probes, Oregon) in 0.2 M KCl as the lineage dye. Observations were made with a Zeiss Universal microscope equipped with a Leitz 50 $\times$ water-immersion objective and a computer-controlled stage using Nomarski optics and ultraviolet epilumination (D.A.K. and C.B.K., submitted). Recording and playback for time-lapse was controlled by a Macintosh II computer (Apple Computer Company) equipped with a Quick Capture board and RasterOps board running software Neuro Video 3.0 (ref. 22) and recording video images onto an Optical Memory Disk Recorder (Panasonic).

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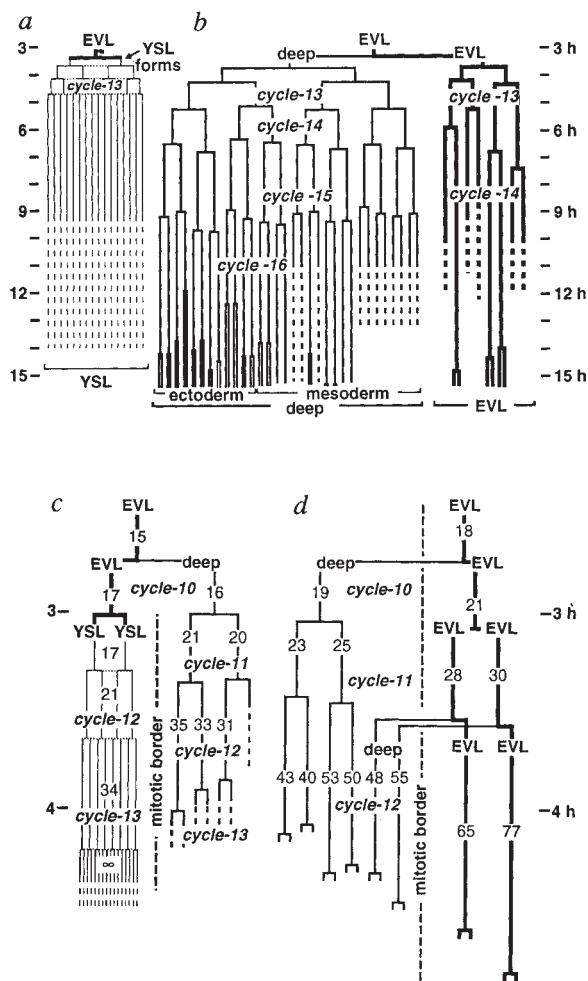


FIG. 2 Changes in cell-cycle length occur during lineage segregations into separate mitotic domains. Mitotic borders (lower panels) are the correlates in these lineage diagrams of spatial borders of the domains in the embryo: after segregations occur, the cycle lengths rapidly diverge across these borders. *a*, YSL mitotic domain recorded from time-lapse Nomarski video microscopy. At division 10, the YSL (thin lines) forms from 'marginal' EVL cells (thick lines) at the blastoderm-yolk margin⁵, and then undergoes usually three (as shown here), but sometimes four or five, synchronous or occasionally metachronous nuclear divisions. After the mitotic arrest, this syncytium extends vegetally during epiboly; the blastoderm follows, eventually covering the yolk cell. Throughout epiboly, the YSL nuclei were never observed to enter mitosis ($n=9$ animals), resembling *Drosophila* domain A and domain B (ref. 1). *b*, Domains of the zebrafish blastoderm. This lineage, from an EVL cell labelled by intracellular injection of tracer dye at cycle 10, produces an EVL and deep sibling; the resultant EVL side of the lineage (thick lines) divides with a slower rhythm than that of the deep side (medium lines). As in *Drosophila* domains α -14N and α -14M (ref. 1), the EVL and deep domains are both asynchronous. But within a clonal subset, the deep cells tend to maintain high synchrony compared to the asymmetric divisions of the EVL cells. Despite the deep lineage producing both ectodermal (neurons and skin) and mesoderm (notochord and muscle) derivatives, there is no hint, considering cycle-length alone, of the cell fate midway through cycle 15. This example is typical of 12 other deep-cell lineages. *c*, Lineage of a marginal EVL cell (thick line) which divides to make a deep cell (medium lines) and a marginal EVL cell which, as it divides, enters the forming YSL (thin lines); immediately afterwards the cell cycle of the YSL becomes faster than that of the deep sublineage. *d*, Typical lineage relationships between EVL and deep cells. Here, an EVL cell divides to produce a cycle-10 EVL/deep sibling pair with the EVL cell (thick lines) having the longer cell cycle. The deep cell (medium lines) gives rise to deep cells only. The EVL produces cycle-11 EVL siblings, each of which produce typically asymmetric cycle-12 EVL/deep sibling pairs (18/23 cases; $P<0.05$). Note the similarities among the cell cycles of all the deep cell cousins. The number in each line indicates the time in minutes for the cell cycle. Abbreviations: EVL, enveloping layer cell; deep, deep cell; YSL, yolk syncytial layer.

cells then pass through cycles 15 and 16, both occurring during gastrulation, their cycles again lengthen, but mother cycle length becomes a poor predictor of daughter cycle length (Fig. 3*d*). EVL cell cycles lengthen during mid-cycle 13, to reach a long cycle 14 that extends through gastrulation (Fig. 3*e*) and beyond (as in Fig. 2*b* for example). The departure from nucleocytoplasmic control seems most dramatic in the YSL, however, which after cycle lengthening during MBT (Fig. 3*c*), enters mitotic arrest during interphase 14 (Fig. 2*a*), like what has been observed in the teleost *Fundulus*⁶. The loss of nucleocytoplasmic control may indicate the requirement for zygotic regulation of the cell cycle, as in cycle 14 of *Drosophila*⁷; indeed, in zebrafish embryos that have been treated with the transcriptional inhibitor α -amanitin, cell-cycle length abnormalities first appear in cycle 14 and 15 (D.A.K., unpublished observations).

The deep cells form all of the embryonic lineages, becoming lineage-restricted to one or another histotype at gastrulation onset^{8,9} when they are in cycle 14 or 15. Up until the end of gastrulation, we found no correlations within this domain between cycle characteristics and eventual cell fate. For example, in the lineage shown in Fig. 2*b*, all of the deep cells divide with similar rhythms through at least the early part of cycle 16, irrespective of whether or not they have involuted into the

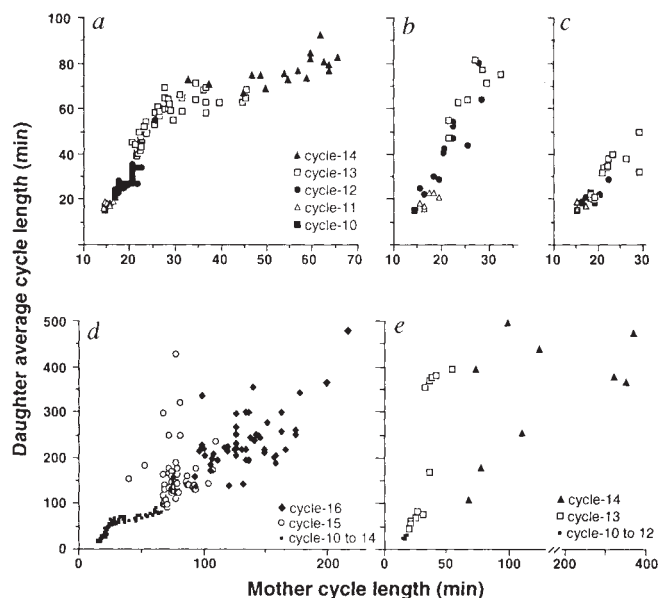


FIG. 3 The end of the MBT period, shown by the loss of cell cycle control by the nucleocytoplasmic ratio. Measurements are combined from 21 embryos, in which the cell cycle lengths for complete mother/daughter pair families were analysed at each division. Each measurement, corresponding to an individual mitosis, is the average cell cycle of the two daughter siblings compared with the cell cycle length of their mother cell. Control of cycle length by the nucleocytoplasmic ratio predicts that the average cycle length of the daughters is a function of that of the mother (see text), and is consistent with the initial close correlations during cycle 11, cycle 12 and early cycle 13 for *a*, the deep-cell domain, *b*, the EVL domain, and *c*, the YSL domain. Individual correlations for the linear portions of *a*, *b* and *c* are highly significant ($P<0.01$). Note that only the early portion of cycle-13 data is presented in *b* (compare with *e*). *d* and *e*, Loss of cycle control by the nucleocytoplasmic ratio is indicated by the loss of the close correlation between mother and daughter. *d*, Cycle lengths of the deep cells plateau at 65 ± 10 min in late cycle 13 and cycle 14 (shown in *a* at larger scale) and then lengthen unpredictably in cycle 15 and 16. *e*, Cycle lengths of the EVL lengthen to 400 ± 100 min in late cycle 13 and cycle 14 cells. The YSL enters a mitotic arrest at about the same time (Fig. 2*a*). Symbols (indicating cycle of the daughters): filled square, cycle 10; open triangle, cycle 11; filled circle, cycle 12; open square, cycle 13; filled triangle, cycle 14; open circle, cycle 15; filled diamond, cycle 16; dots in *d*, cycle 10 to 14; and dots in *e*, cycle 10 to 12.

hypoblast, that is, irrespective of whether they later give rise to ectodermal or mesodermal fates¹⁰. Thus even though mitotic domains arise at equivalent stages in zebrafish and *Drosophila*, there may be important differences between them; more domains are present in the fly, and they correspond to the fate map of the embryo¹. There appears to be no hint of different mitotic domains in the fish gastrula that correspond to different embryonic fate map regions.

In a sense, there is broad correspondence between fate and mitotic domain: the YSL and EVL mitotic domains are separately mapped lineage compartments that will generate extra embryonic fates. These lineage restrictions occur either coincidentally with or just after the formation of the mitotic domains⁸. Again, there may be a key difference between mitotic domains in zebrafish and *Drosophila*: in the fly, the mitotic domains are forming as cell commitments are being made^{11,12}, and probably reflect these commitments to fate. This appears unlikely for at least the zebrafish EVL, for these cells transplanted singly into the deep cell domain can switch fate well after mitotic domains are established¹³. Thus, the mitotic domains in the zebrafish may have little significance with respect to cell commitment.

Interestingly, however, each mitotic domain plays a different role in the embryo's first morphogenetic movement, epiboly, that begins in the late blastula¹⁰, just after the domains themselves form. The YSL begins to expand actively towards the vegetal pole, and, at least in the teleost *Fundulus*, adherent junctions are present between it and bordering EVL cells^{14,15} such that the YSL can tow the EVL along behind it^{16,17}. Simultaneously, motile deep cells undergo radial intercalations¹⁰,

similar to those in *Xenopus*¹⁸, which participate in generating the uniformly thick blastoderm that is present when gastrulation begins. It may well be that early decisions expressed during and immediately after the zebrafish MBT period regulate the processes of epiboly and gastrulation^{19,20}, the morphogenetic tasks that the embryo is directly facing, rather than the commitment of lineages, as would seem to be the case in *Drosophila*. □

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Retinoic acid alters hindbrain *Hox* code and induces transformation of rhombomeres 2/3 into a 4/5 identity

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It has been suggested that *Hox* genes play an important part in the patterning of limbs^{1–3}, vertebrae^{4–6} and craniofacial structures^{5,7–9} by providing an ordered molecular system of positional values, termed the *Hox* code^{10,11}. Little is known about the nature of the signals that govern the establishment and regulation of *Hox* genes, but retinoic acid can affect the expression of these genes in cell lines^{12–14} and in embryonic tissues^{2,11,15–17}. On the basis of experimental and clinical evidence, the hindbrain and branchial region of the head are particularly sensitive to the effects of retinoic acid^{15,18–21}, but the phenotypes are complex and hard to interpret, and how and if they relate to *Hox* expression has not been clear. Here we follow the changes induced by retinoic acid to hindbrain segmentation and the branchial arches using transgenic mice which contain *lacZ* reporter genes that reveal the endogenous segment-restricted expression of the *Hox-B1* (*Hox-2.9*), *Hox-B2* (*Hox-2.8*) and *Krox-20* genes. Our results show that these genes rapidly respond to exposure to retinoic acid at preheadfold stages and

undergo a progressive series of changes in segmental expression that are associated with specific phenotypes in hindbrain of first branchial arch. Together the molecular and anatomical alterations indicate that retinoic acid has induced changes in the hindbrain *Hox* code which result in the homeotic transformation of rhombomeres (r) 2/3 to an r4/5 identity. A main feature of this rhombomeric phenotype is that the trigeminal motor nerve is transformed to a facial identity. Furthermore, in support of this change in rhombomeric identity, neural crest cells derived from r2/3 also express posterior *Hox* markers suggesting that the retinoic acid-induced transformation extends to multiple components of the first branchial arch.

In the process of defining the regulatory elements required for spatially restricted domains of *Hox-B* expression, we have produced lines of mice carrying *Hox/lacZ* transgenes that reproduce the endogenous pattern of *Hox-B1* expression, including its ability to respond to retinoic acid²² (H.M. *et al.*, manuscript in preparation). In addition, we have mapped regions responsible for the rhombomeric expression of *Hox-B2* in r4 and second arch neural crest (M.H.S. *et al.*, unpublished), and produced *lacZ* transgenic lines that reflect the endogenous patterns of *Krox-20* expression in hindbrain segmentation²³. These transgenic mice provide a convenient set of cellular markers for segmental expression in r3/4/5, the neural crest and motor nerves associated with this region. To investigate the effects of retinoic acid on *Hox* expression and segmental patterning in the hindbrain, we have exposed mouse embryos at several early stages to concentrations of retinoic acid (20 mg per kg) that are known to induce craniofacial abnormalities^{16,17}. The *Hox/lacZ* transgenic lines were used to monitor molecular and anatomical changes in segmentation throughout a detailed time course. The results were independently confirmed by *in situ* hybridization of the endogenous genes at selected stages (data not shown). Our results focus on the reproducible phenotype generated by retinoic acid treatment at 7.5 days post-coitum (d.p.c.).

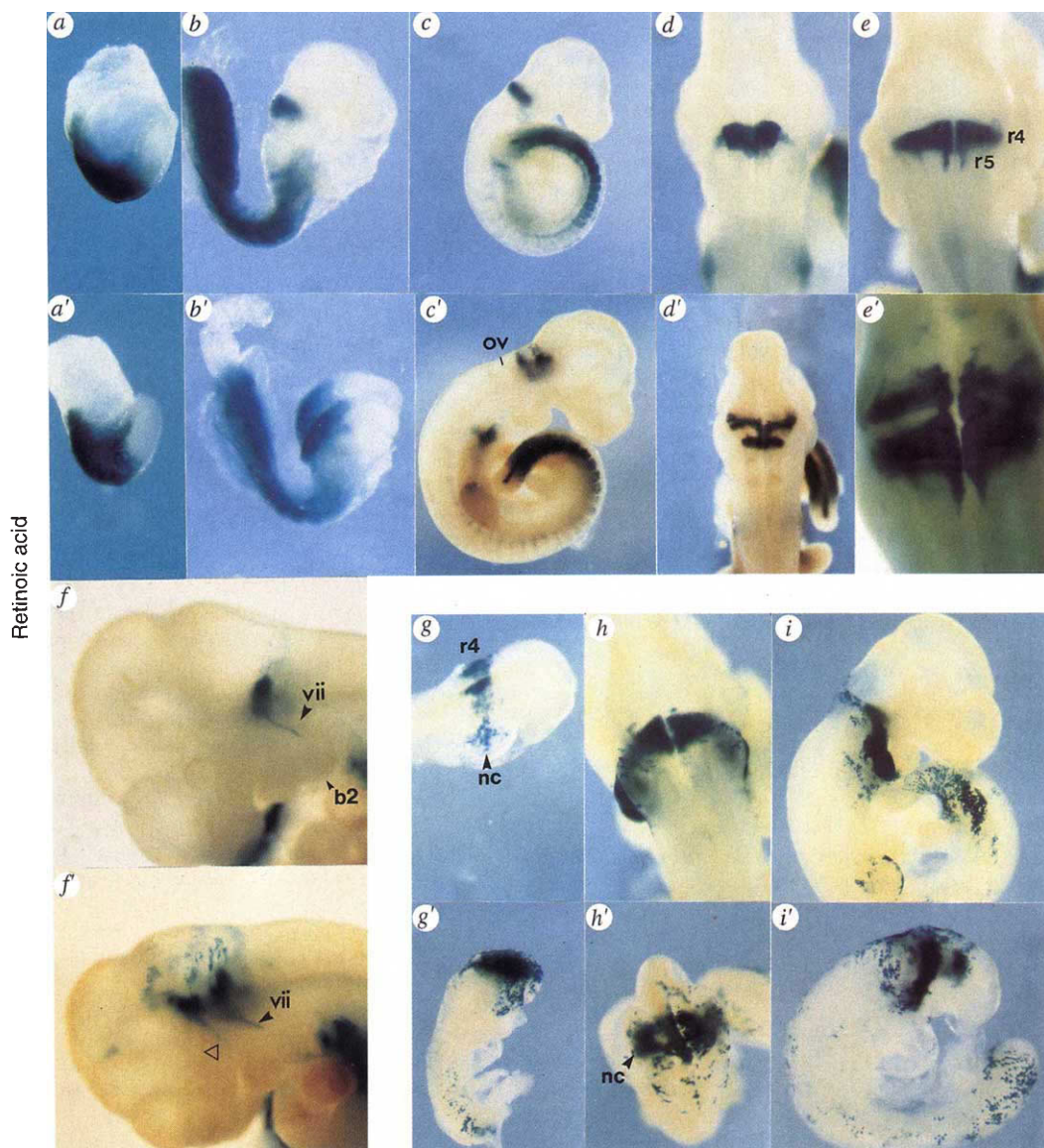
The *Hox-B1/lacZ* transgene, like the endogenous gene^{24–27}, is initially activated in a broad domain at preheadfold stages,

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with an anterior limit mapping to the future r3/4 boundary. There is a progressive restriction of the anterior domain of expression which is limited to rhombomere 4 in the hindbrain by 9.5 d.p.c. (Fig. 1*a-d*). At 10.5 d.p.c. *Hox-B1* is also expressed in the facial motor nerve (vii), whose β -galactosidase-stained axons exit the hindbrain from r4 and innervate the second branchial arch (Fig. 1*f, f'*). Staining also appears adjacent to the midline in r5 (Figs 1*e* and 2*a*), a region that could correspond

with the location of motor neuron cell bodies. To visualize the positions of all efferent neurons that exit via the vii/viii nerve, we therefore applied the retrograde axonal tracer DiI to the exit point in r4. This revealed that in normal embryos r5 motor neurons are in two subsets: a medial group of 10–20 neurons whose axons project anteriorly along the side of the floor plate, and a larger lateral group whose axons extend laterally before turning anteriorly into r4 (Fig. 2*c, f*). Both groups belong to the

FIG. 1 Retinoic acid (RA) induces *Hox* expression of r4 and second arch neural crest markers in r2 and first branchial arch. *Hox-B1* response to RA. *a-f*, The normal pattern of expression found in untreated embryos carrying the *Hox-B1/lacZ* transgene. (*a*) Lateral view of 7.75 d.p.c. embryo showing expression of *Hox-B1* extending along the neural epithelium to the presumptive future r4 region of the hindbrain. Anterior expression becomes restricted to r4 and gradually regresses posteriorly along the spinal cord as seen in the lateral views at 8.5 d.p.c. (*b*) and 9.5 d.p.c. (*c*). The dorsal view of the 9.5 d.p.c. embryo (*d*) clearly shows that the later phase of *Hox-B1* expression is restricted to r4 and a small population of neural crest cells migrating laterally from the region. At 10.5 d.p.c. (*e*) a small domain of expression appears along the ventral midline of r5 adjacent to the floorplate and represents the cell bodies of the facial nerve. Below each panel is a similar transgenic embryo at the same stage and plane of view which has been exposed to RA (*a'-f'*). At 7.75 d.p.c. (*a'*) expression has expanded to a more anterior domain. By 8.5 d.p.c. (*b'*) expression posterior of r4 begins to regress as in the control, but there is a broadened domain extending from r4 into the midbrain. This anterior domain eventually resolves at around 9.5 d.p.c. (*c', d'*) to give a two-stripe pattern of expression in r2 and r4. There is also expression in the motor nerve cell bodies along the midline of r3 at 9.5 d.p.c. (*d'*) and r3+r5 at 10.5 d.p.c. (*e'*). The transgene is expressed in both treated and untreated embryos in the facial motor nerve (vii) exiting r4 and innervating the second branchial arch (b2) (*f, f'*). In addition to a normal facial motor nerve, RA induces the expression of the *Hox-B1/lacZ* transgene in a second motor nerve that exits from r2 and innervates the first branchial arch indicated by the open arrow head (*f'*). This is the normal position of the trigeminal motor nerve. By 10.5 d.p.c. there is a complete duplication of the normal r4/5 pattern of expression in r2/3. ov, Position of the otic vesicle; r, rhombomere. *Hox-B2* response to RA. *g-i*, Normal staining patterns of a *Hox-B2/lacZ* transgene which is primarily expressed in r4 and neural crest migrating into the second branchial arch. Some patchy expression can be found in cells of the posterior ectoderm. *g*, Lateral view at 8.5 d.p.c.. *h*, Dorsal and *i*, lateral views of a 9.5 d.p.c. embryo. *g'-i'*, In embryos exposed



to RA the early r4 expression domain expands at 8.0 d.p.c. (*g'*) as observed using the *Hox-B1* markers. Neural crest (nc) streams from the entire expanded region as seen in the dorsal view at 8.5 (*h'*). The early expression is modified and becomes restricted to r2 and r4 and their migrating crest populations which is seen in a lateral view of a 9.0 d.p.c. embryo (*i'*).

METHODS. Permanent lines of transgenic mice were produced by microinjection into fertilized eggs from F_1 backcrosses (CBA $\times$ C57) and transferred to pseudopregnant females^{29,30}. The genomic constructs were isolated from the mouse *Hox-B1* and *B2* genes into which the *E. coli* β -galactosidase reporter gene was inserted to create fusion proteins (H.M. and M.H.S., manuscripts in preparation). Embryos were stained for β -galactosidase activity in whole preparations as described previously³¹. All-trans retinoic acid (25 mg ml⁻¹ in dimethylsulphoxide) was diluted 1/10 in sesame seed oil and ~200 μ l of this was given to pregnant females at 7.5 d.p.c. by gavage for a final dose of 20 mg kg⁻¹ as previously described¹⁷.

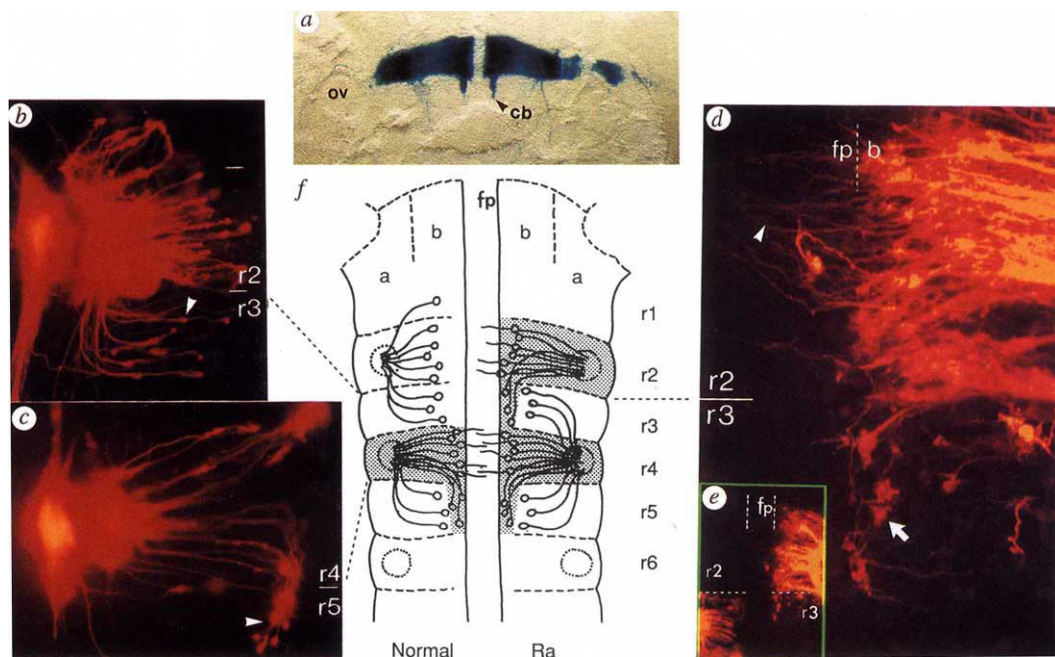
facial branchial motor nerve. In addition to the facial motor neurons in the basal plate of r4, retrograde tracing from the vii/viii nerve exit point revealed a segment-specific group of sensory efferent neurons belonging to the vestibuloacoustic nerve whose primary dendrites extend across the midline. Later in development the cell bodies of these neurons translocate into the other side of the brain (H. Simon and A.L., manuscript submitted). This neuronal organization is unique to the r4/5 region and in r2/3 we found that trigeminal efferent neurons traced from the exit point in r2 are more laterally placed, with their axons all extending laterally through the neuroepithelium (Fig. 2b, f). Expression of the *Hox-B1/lacZ* transgene in r4 and r5 therefore co-localizes with two distinct groups of neuronal cells that are not present in r2 and r3.

Retinoic acid rapidly alters *Hox-B1* expression¹⁷, and 4 h after application there is an anterior shift of the expression boundaries in both mesoderm and neural ectoderm (Fig. 1a'). At 8.5 d.p.c. (20 h after treatment with retinoic acid) in both treated and control embryos post-otic domains of expression regress posteriorly, but in treated embryos the pre-otic domain is very broad and extends from r4 into the midbrain (Fig. 1b, b'). But, this broadened domain is transient and by 9.5 d.p.c. becomes progressively restricted to two stripes in the hindbrain corresponding to r2 and r4 (Fig. 1c, c'). Between 9.5–10.5 d.p.c. the retinoic acid-induced ectopic expression of *Hox-B1* also appears in the motor nerve associated with r2/3. The cell bodies along the

midline of r2 and r3 now express the transgene, as well as the nerve that exits r2 and innervates the first branchial arch (Fig. 1d'–f'). The positions of efferent neurons in r2 and r3 and the courses through the neural epithelium of their axonal projections revealed by DiI tracer injection, show alterations consistent with their transformation to a more posterior phenotype. In r2, medial efferent neurons extend processes across the midline as normally seen only in r4. The neurons in r3 now include a medial, anteriorly extending group that resembles those of r5 (Fig. 2d, e, f); these and the medial branchial motor neurons of r2 exhibit strong *Hox/lacZ* staining, reduplicating the pattern of r4/5. The balance of r3 motor neurons are revealed by tracer injections into the seventh nerve exit point (r4) rather than the fifth (r2), indicating that these too may be 'facial' motor neurons²⁸ (Fig. 2d, e). A diagrammatic summary of the nerve pathways of normal and retinoic acid-treated embryos is shown (Fig. 2f). These data show that there has been a posterior transformation of the trigeminal territory to a facial/vestibuloacoustic identity, and illustrates that retinoic acid induces changes in *Hox-B1* expression that lead to the duplication of the r4/5 patterns in r2/3 without changing the normal r4/5 domains.

If this represents a true transformation, then other aspects of the segmental *Hox* code should also be altered. Therefore we examined another *Hox/lacZ* transgenic line that reproduces the subset of *Hox-B2* expression found in r4, and its neural crest migrating into the second branchial arch (Fig. 1g–i). At

FIG. 2 *Hox-B1* marks the neurons of the facial motor nerve in r4/5 and retinoic acid (RA) induces a duplication of the facial motor nerve in trigeminal territory. a, A flat mounted hindbrain preparation of an untreated 10.5 d.p.c. embryo highlighting the staining of the *Hox-B1/lacZ* transgene in the facial motor nerve exiting r4, and along the midline in r5 adjacent to the floor plate. b, Cell bodies of the trigeminal nerve in r3 of a normal embryo are not located at the ventral midline but are distributed more laterally (arrowhead) and send their axons directly lateral before turning anteriorly and crossing into r2. c, DiI retrograde tracing of the facial motor nerve in a normal embryo shows that the cell bodies in r5 (indicated by the white arrow) are located along the midline and project their axons anteriorly into r4. The transgene staining is co-localized with the cell bodies of the facial nerve. In b and c one half of the hindbrain is shown with the floor plate and ventral midline to the right, and the dorsal/lateral edge to the left. d, In RA-treated embryos labelled with an injection of DiI at the exit point in r2, the cell bodies of the efferent axons in r3 have the characteristics of the facial nerve (in the normal r5) in that they are located along the lateral border of the floor plate and initially have a direct rostral trajectory (large arrow). r2 neurons also extend processes across the floor plate (small arrow head) as in the normal r4. In this specimen, DiI was injected at both the r2 exit point (on the right side of the embryo) and at the r4 exit point on the left side. e, Inset showing both sides of the specimen in d. Note that lateral motor neurons in r3 are traced by dye applied to the r4 exit point (and not r2 as in normal embryos); thus the r3 territory is shared by the rostral r4-labelled cells (left side) and the caudal r2-labelled cells (right side). Confocal Z-series maximum brightness projection displayed in false colour LUT. f, Diagrammatic summary showing the positions of the axons, cells bodies and the *Hox-B1/lacZ* expression (stipple) in normal and



RA-treated flat-mounted 10.5 d.p.c. mouse embryo hindbrains; it appears that the trigeminal nerve has been transformed into a facial identity. ov, Otic vesicle; cb, cell bodies of the facial motor neurons; fp, floor plate; b, basal plate; a, alar plate.

METHODS. Flat-mount slide preparations were made by isolating the region of the hindbrain concerned, cutting at the dorsal midline, opening out and flattening the tissue, and mounting in glycerol. One half was stained with β -gal to verify phenotype, and in the other half efferent cranial nerves emerging from the hindbrain were traced in paraformaldehyde fixed embryos 10–10.5 d.p.c. by injecting small volumes DiI_{C₁₈} (1, 1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, molecular probes, 5 mg ml⁻¹ in dimethylformamide) at their exit points. After 24 h at room temperature to allow diffusion along axonal membranes, hindbrains were dissected from the embryos, flat-mounted in fixation buffer and viewed both under green excitation on a fluorescence microscope (Zeiss, set 15) and a confocal microscope (BioRad) using the 514 nm laser line.

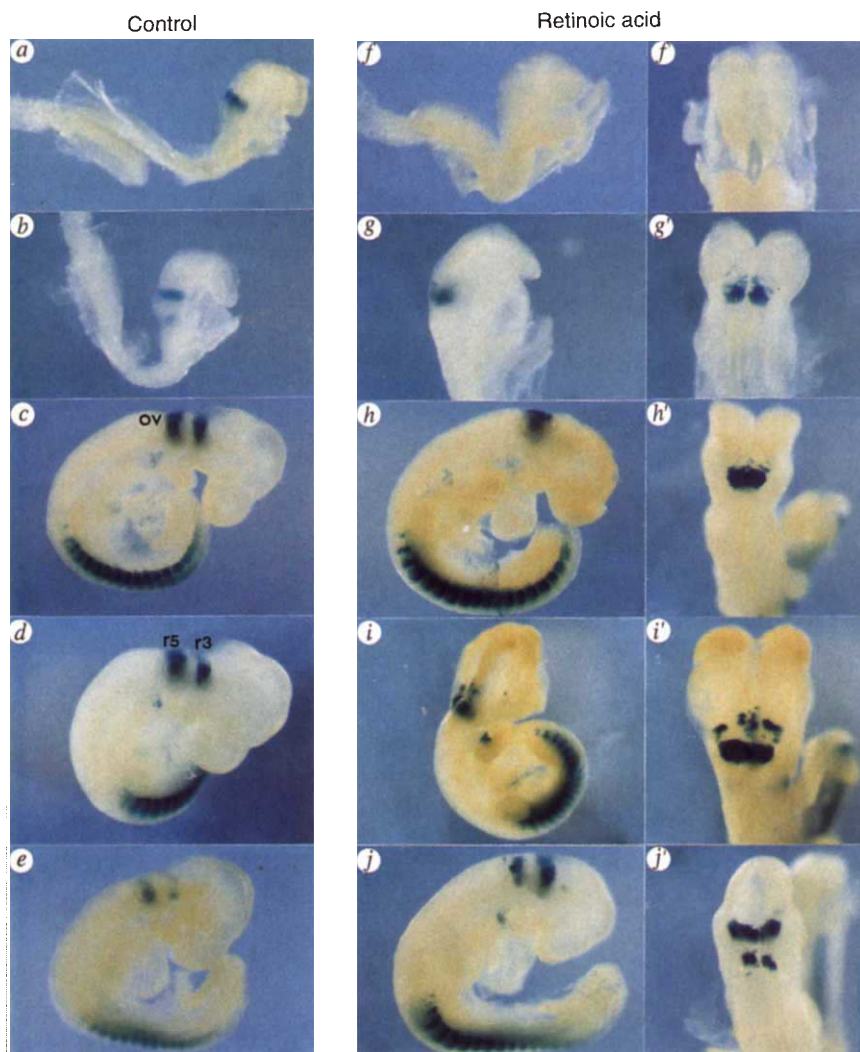
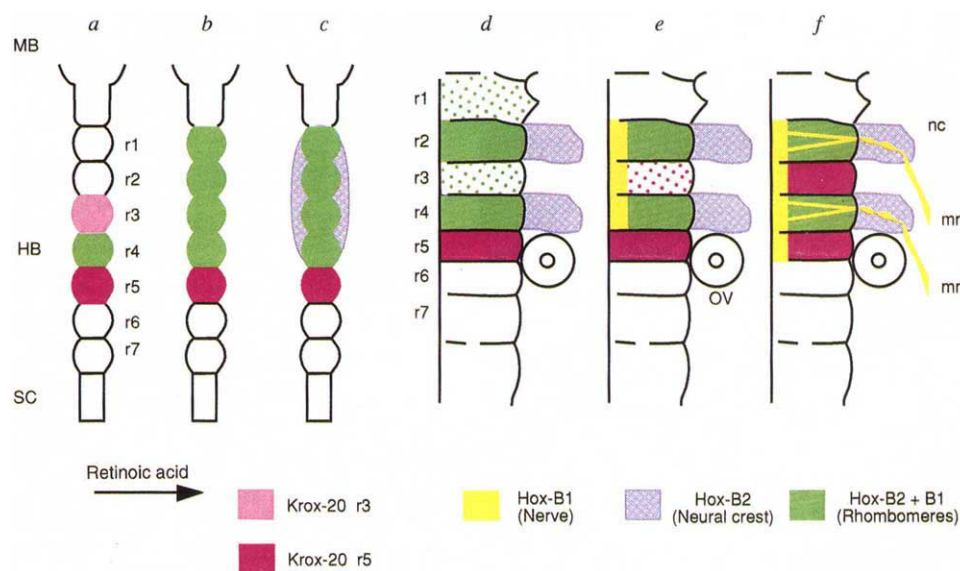


FIG. 3 Retinoic acid represses the early r3 stripe of *Krox-20* expression and reactivates expression at later stages. *a-e*, Lateral views of the temporal and spatial patterns of expression reflected by the *Krox-20* gene in control transgenic embryos. At 8.25 d.p.c. staining first appears in r3 (*a*) and after a few hours is joined by staining in r5 (*b*). This pattern persists up to 9.5 d.p.c. (*c*) when both stripes of expression are at their strongest. At 10.0 d.p.c. (*d*) *Krox-20* expression begins to be downregulated in r3 and is followed, at 10.5 d.p.c. (*e*) by the downregulation of expression in r5; *f-j'* (lateral views) and *f'-j'* (dorsal views) show that retinoic acid treatment alters this pattern. The gene is not initially activated in r3 at 8.25 d.p.c. as shown in *f* and *f'*. At 8.5 d.p.c. (*g, g'*) expression appears in r5 and not in r3. At a stage when both endogenous stripes would be present and at their highest level, the treated embryos express the gene only in r5 (*h, h'*). At 10.0 d.p.c., expression is beginning to appear in r3 (*i, i'*) and this pattern persists until 10.5 d.p.c. (*j, j'*) when expression of *Krox-20* begins to be downregulated in r5, but remains at a high level in r3. ov, Otic vesicle; r3, r5 rhombomeres 3 and 5.

FIG. 4 Temporal changes in the hind-brain *Hox* code induced by retinoic acid.

a, Diagrammatic representation of the normal pattern of expression of *Krox-20*, *Hox-B1*, and the r4 domain of *Hox-B2* in untreated 8.5 d.p.c. embryos. When a single dose of RA (20 mg kg^{-1}) is given to 7.5 d.p.c. embryos the patterns of expression undergo a series of modifications between 8.5–10.5 d.p.c., as indicated by the series of sketches. *b*, Initially *Krox-20* does not appear in r3, but does appear in r5. *Hox-B1* and *Hox-B2* are expressed in r4 through an expanded domain which extends to the midbrain. *c*, Neural crest migrating from this expanded domain expresses *Hox-B2*, instead of just the cells migrating from r4 in control embryos. *d*, Around 9.0 d.p.c. expression of *Hox-B1* and *Hox-B2* declines (as indicated by dots of colour) in r1 and r3 and there are two distinct populations of neural crest cells adjacent to r2 and r4 expressing *Hox-B2*. *e*, By 9.5 d.p.c. *Krox-20* begins to appear in r3 (dots), and there is staining of *Hox-B1* along the midline of r2/3/4 which corresponds to cell bodies of both the normal facial nerve and the transformed nerve which will exit from r2. *f*, By 10.5 d.p.c. there is a complete duplication of the r4/5 patterns of expression of *Hox-B1*, *Hox-B2* and *Krox-20* in r2/3, including the associated motor nerves and neural crest. Cell bodies in r2–r5 and both motor nerves



express *Hox-B1*. *Krox-20* is highly expressed in r3 and r5. These changes show that RA has altered the *Hox* code in a manner that leads to the posterior transformation of r2/3 in the first branchial arch. MB, midbrain; HB, hindbrain; SC, spinal cord; OV, otic vesicle; mnVII, facial motor nerve; nc, neural crest.

8.0 d.p.c., retinoic acid causes an expansion of the r4 stripe in an anterior direction into the midbrain, and at 8.5 d.p.c. there is also expression in neural crest cells migrating from this broadened domain (Fig. 1g', h'). Between 9.0–9.5 d.p.c., these patterns become refined with a progressive restriction leading to the appearance of stripes of expression in r2 and r4 and a stream of crest cells migrating from each (Fig. 1i'). These dynamic changes in spatial expression are identical to those observed with *Hox-B1*, therefore the combination of *Hox* genes expressed in r4 (ref. 10) is reproduced in r2 upon exposure to retinoic acid.

The *Hox-B1* experiments showed that a marker for r5 (expression in neural cell bodies) was activated in r3 and we wanted to examine whether there were other changes to segmental expression in r3. We have used a line of transgenic mice that accurately reveals the normal spatial and temporal patterns of *Krox-20* expression in r3 and r5 (Fig. 3a–e; ref. 23). In initial stages, as reported for both *Xenopus*¹⁵ and mouse^{16,17} embryos treated with retinoic acid, we find that although there is a clearly defined segment in the r3 position, the r3 stripe of *Krox-20* expression is absent. There is no change in the r5 pattern of expression (Fig. 3f–h, f'–h'). We found, however, that these patterns are modified at later stages when a new domain of *Krox-20* emerges in r3 (Fig. 3i, j, i', j'). Taken together with our other data, this pattern represents a duplication of the r5 expression in r3, rather than a delay in the onset of r3 expression.

The temporal changes in segmental expression and anatomical variation induced by retinoic acid are summarized in Fig. 4. Initially retinoic acid inhibits the appearance of markers in the r1–3 region, such as *Krox-20*, and expands those of the r4 in an anterior direction to the midbrain. There are no apparent changes in the *Hox* code in the r5–7 region. Accompanying the expanded domain of r4 expression, neural crest cells migrating from this region also express r4 markers. An important finding is that these changes are not stable; expression of r4 markers continues in r2 but they are specifically downregulated in r1 and r3. Together with this downregulation there is an activation of r5 markers in r3. Owing to the fact that there are underlying molecular mechanisms that modify these early retinoic acid-induced patterns, the net effect of retinoic acid application to preheadfold-stage mouse embryos is a duplication of the r4/5 *Hox* code in r2/3, which ultimately results in a homeotic transformation of rhombomeric phenotypes. It is important to note that segmentation in the hindbrain has a role outside the neural epithelium in patterning the branchial region by way of the neural crest, and the experimental²⁹ and clinical²⁰ dysmorphologies induced by retinoic acid frequently include the appearance of structural alterations in the first branchial arch. Because we observe changes in *Hox* expression in neural crest and motor nerves as well as in the early neural epithelium, multiple groups of cells that will enter and populate the first branchial arch may have adopted a second branchial arch identity. Therefore some of the clinical retinoic acid-treated phenotypes²⁰ could be explained by alterations in *Hox* expression similar to those reported here.

The early retinoic acid-induced ectopic expression of the r4 *Hox* code in a broad anterior region of the hindbrain does not permanently result in this entire region adopting an r4 identity, as has been proposed^{16,17}. Rather, our results imply that at the preheadfold time of treatment (7.5 d.p.c.), a certain degree of regional variation has already been specified at the level of rhombomere pairs; retinoic acid is unable to override the specification choice of an odd- or even-numbered character. This is implied by the subsequent downregulation of the *Hox-B1* and *B2* domains specifically in r1 and r3. An important point in these experiments is that retinoic acid application at 7.5 d.p.c. causes the rhombomere transformations, but the rhombomeres themselves appear normal in size. Hence retinoic acid does not seem to alter the relative growth and elaboration of rhombomeres, but induces changes in their identity. But we have noted that treatments of retinoic acid at even earlier stages of

embryogenesis (7.0 d.p.c.) causes more extensive alterations in midbrain/forebrain structures and result in a compression of the most rostral rhombomeres (r1–r3) in the hindbrain, as observed in other vertebrate embryos^{15,19}. Initial analysis of *lacZ* staining patterns in such embryos suggests that, despite this compression, there has also been a duplication of r4 markers in the r2 region. Therefore differences in the timing of retinoic acid exposure can independently alter processes of growth and regional identity in the hindbrain, which could explain much of the phenotypic variation in retinoic acid-treated embryos.

Note added in proof: We have adopted the revised nomenclature for vertebrate *Hox* genes suggested by the Beechwood Ridge Nomenclature Committee³² which replaces *Hox-2* with *Hox-B* as a complex and, individually *Hox-2.9* = *Hox-B1* and *Hox-2.8* = *Hox-B2*. □

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Bone and haematopoietic defects in mice lacking *c-fos*

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THE proto-oncogene *c-fos* is the cellular homologue of *v-fos* originally isolated from murine osteosarcoma¹. *Fos* protein is a major component of the AP-1 transcription factor complex, which includes members of the *jun* family². Stable expression of *c-fos* in mice has been demonstrated in developing bones and teeth, haematopoietic cells, germ cells and in the central nervous system^{3–11}. It has been proposed that *c-fos* has an important role in

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signal transduction, cell proliferation and differentiation¹²⁻¹⁵. We have previously demonstrated that overexpression of *c-fos* in transgenic and chimaeric mice specifically affects bone, cartilage and haematopoietic cell development¹⁶⁻²⁰. To understand better the function of *c-fos* *in vivo*, we used gene targeting in embryonic stem cells to generate cells and mice lacking *c-fos*. Here we report that heterozygous *fos* $+/-$ mice appear normal, although females exhibit a distorted transmission frequency. All homozygous *fos* $-/-$ mice are growth-retarded, develop osteopetrosis with deficiencies in bone remodelling and tooth eruption, and have altered haematopoiesis. These data define the c-Fos protein as an essential molecule for the development of specific cellular compartments.

The targeting vector used to disrupt the *c-fos* gene in embryonic stem (ES) cells is shown in Fig. 1a. In two independent experiments, four cell clones were targeted at the *c-fos* locus (for example, D2-25, D22; Fig. 1b). In addition, cells were isolated that completely lacked Fos (for example, D22-1 and D22-2 in Fig. 1b) and were viable, showing no abnormalities

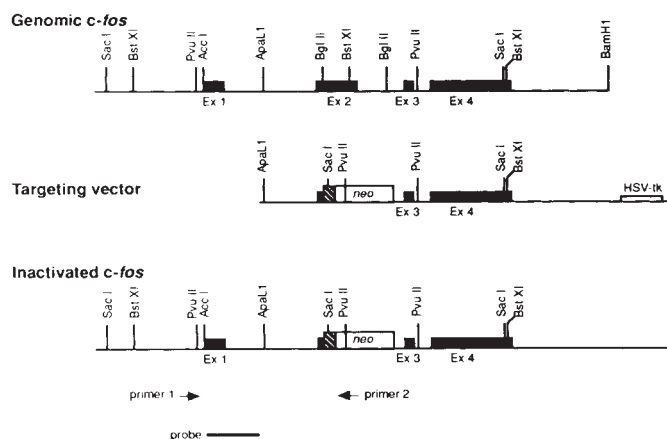
in vitro (data not shown). A similar observation was made in ES cells lacking c-Jun²¹.

Several chimaeric mice generated with D2-25 and D22 cells (derived from two independent experiments) transmitted the targeted *c-fos* allele to their offspring. Mice heterozygous for the *fos* mutation appear normal. The *fos* genotypes of offspring derived from heterozygote intercrosses are shown in Fig. 1c. Using Southern blot analysis, we have genotyped 383 F_2 offspring and 54 F_2 fetuses and found a distribution of 38% $+/+$, 50% $+/-$ and 12% $-/-$ mice (Table 1). Because no

FIG. 1 Homologous recombination at the *c-fos* locus in mouse embryonic stem (ES) cells. **a**, Top panel, structure of the targeting vector and partial restriction map of the *c-fos* locus before and after targeted integration. The targeting vector contains *c-fos* sequences (derived from 129/Sv mice) from position 459 of the *c-fos* coding region to the *Bam*HI site which is ~800 base pairs (bp) downstream of the *c-fos* polyadenylation signal. The neomycin-resistance gene (*neo*) was fused in-frame to *c-fos* replacing part of the second exon and intron (Bg/II fragment), thereby deleting 483 bp of *c-fos* and introducing additional *Sac*I and *Pvu*II sites which help the identification of the correct targeting event. Bottom panel, the PCR product and the expected fragment sizes of wild-type and mutant *c-fos* alleles after digestion with *Bst*XI, *Pvu*II or *Sac*I. **b**, Southern blot analysis of ES cell clones containing single or double disrupted alleles of the *c-fos* gene. Genomic DNA from wild-type D3 cells ($+/+$), single targeted ES cell lines, 2-25 and 22 ($+/-$) and double inactivated ES cell clones 22-1 and 22-2 ($-/-$) was digested with either *Bst*XI, *Pvu*II or *Sac*I and analysed by Southern blot hybridization using a 5' external probe. No additional rearrangements of the targeted locus were detected using an internal probe. The sizes of the DNA fragments are indicated in kilobases (kb). **c**, Southern blot analysis of progeny derived from two heterozygote intercrosses. Tail DNA from 7-day-old mice was digested with *Pvu*II and hybridized with a 5' external probe. The genotype at the *c-fos* locus is indicated below each lane. The sizes of the DNA fragments are indicated in kb.

METHODS. A 3.0 kb *Hind*III-*Sac*I fragment from clone C49/105, which contains a 3.4 kb *Sac*I fragment of 5' sequence of *c-fos*, was subcloned into Sp73. A 483 bp *Bg*/II fragment of *c-fos* was deleted from this clone and replaced with a neomycin resistance gene (*neo*)²⁷. The *neo* gene lacked a promoter and an ATG start codon, but contained a TGA stop codon and a synthetic polyadenylation site fused in-frame to the *c-fos* coding sequence. This clone, designated C54/7, was partially digested with *Sac*I and a 6.8 kb fragment was further digested with *Bam*HI to which a 1.5 kb *Sac*I-*Bam*HI fragment of 3' sequence of *c-fos* was fused to give clone C56/20. For negative selection²⁸, the HSV-tk gene with its own promoter was inserted into the *Not*I site of the polylinker downstream of the *c-fos* 3' *Bam*HI site. The tk gene is in the same 5' to 3' orientation as the *c-fos* gene. This construction should lead to a fusion of 53 N-terminal amino acids of the c-Fos protein to the fifth amino acid of the Neo protein with 12 additional amino acids derived from the linker sequence. The final targeting vector was linearized with *Apa*LI and purified before electroporation into feeder-dependent D3 ES cells²⁹. Selection conditions for targeting of the first *c-fos* allele were 0.3 mg ml⁻¹ G418 and 2 μ M gancyclovir. For PCR screening, primer 1 is localized in the TATA box region of the *c-fos* gene and primer 2 in the 5' end of the *neo* gene. Using these primers, the correct homologous recombination event yielded a 1.3 kb diagnostic PCR fragment. Eleven colonies were positive by PCR analysis, of which four were targeted as analysed by Southern blots. For Southern blot analyses, we used a 400 bp *Acc*I-*Apa*LI fragment not present in the targeting vector as a 5' external probe. For targeting of the second allele³⁰ the G418 concentration was increased to 1.0 mg ml⁻¹. Chimaeric mice were generated by microinjection of D2-25 and D22 cells into blastocysts derived from C57BL/6 mice. A total of 40 chimaeras were born of which 34 were males. Test breeding of 17 chimaeric males to C57BL/6 females showed that 13 out of 13 (for D2-25) and 3 out of 4 (for D22) males transmitted the targeted *c-fos* allele to their offspring at a normal frequency.

a Inactivation of *c-fos*

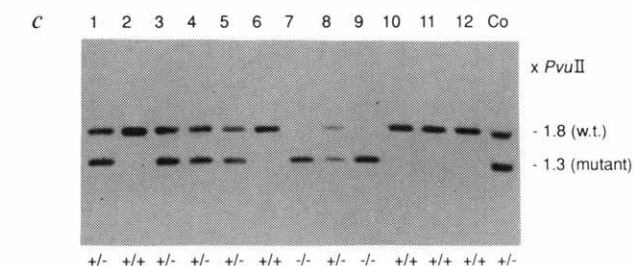
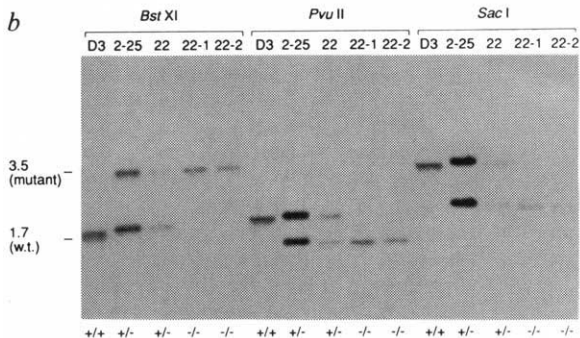


Expected fragment sizes:

PCR: 1.3 kb

Southern blot:

*Bst*XI: w. t. (1.7 kb), mutant (3.5 kb)
*Pvu*II: w. t. (1.8 kb), mutant (1.3 kb)
*Sac*I: w. t. (3.3 kb), mutant (2.0 kb)



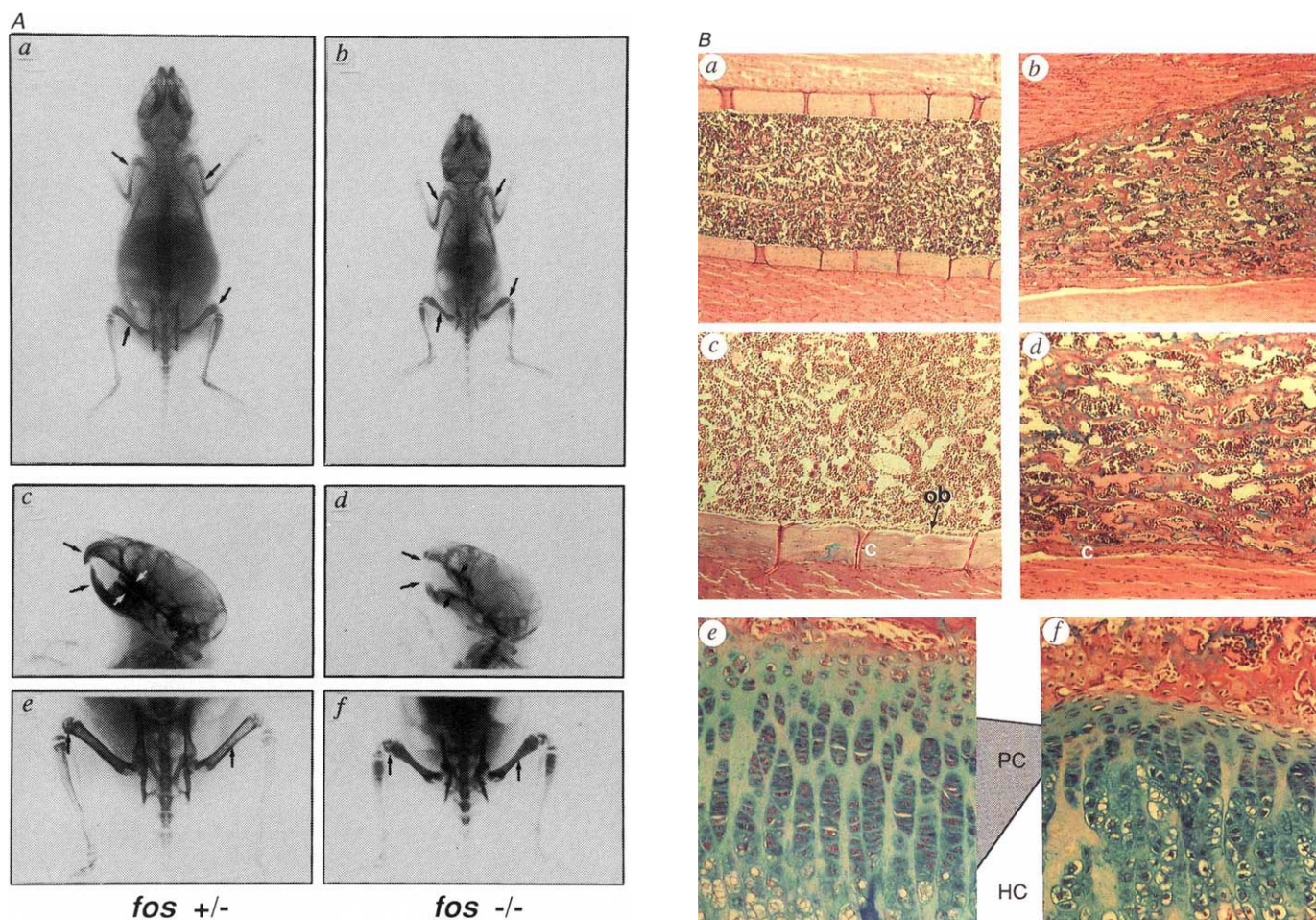


FIG. 2 A, X-ray analysis of a 4-week-old heterozygous *fos* ^{+/+} mouse (a, c, e) and a homozygous *fos* ^{-/-} littermate (b, d, f).

Mutant mice are severely growth retarded compared to a normal heterozygote (a, b). Mandibular and maxillary incisors and molars are not present in *fos* ^{-/-} mice (arrows in c and d). Alterations on the long bones include the absence of a bone marrow cavity and an enlargement of the epiphyses and metaphyses in mutant mice (arrows in a, b, e, f). B, Histological analysis of bone defects in wild-type (a, c, e) and homozygous *fos* ^{-/-} littermates (b, d, f). a–d Longitudinal sections through the femurs. Note the abundance of calcified trabeculae present in the bone marrow cavity of the mutant mice (b, d). The cortical bone in *fos* ^{-/-} mice is poorly developed compared to that of the wild-type littermate (c, d) and there is an absence of osteoblastic cells lining the cortical bone. e, f, Longitudinal sections through the epiphyseal growth plate of the proximal tibiae. The growth plate in mutant mice appears highly disorganized in contrast to that in wild-type mice. In addition, the zone of proliferating cartilage (PC) is drastically reduced in mutant mice with a corresponding increase in the relative size of the hypertrophic chondrocyte zone (HC). c, Cortical bone; ob, osteoblastic cells. C, Histological analysis of teeth in wild-type (a, c) and homozygous *fos* ^{-/-} (b, d) littermates. a, b, Coronal section of maxillary bones showing normal incisor development in wild-type mice in contrast to mutant mice in which incisor eruption is impaired by excessive bone growth. c, d, Sagittal section through the maxillary molars showing complete tooth eruption in wild-type mice, whereas the teeth in mutant mice fail to erupt through the gum because of the persistence of bone. b, Bone; i, roots of upper incisors; m, molars.

METHODS. Mice were killed by cervical dislocation and tissues were fixed in fresh 3.7% paraformaldehyde for 24 h, followed by acid demineralization in 10% formic acid for 24 h, dehydration and embedding in paraffin. All sections are 5 μ m paraffin sections, stained

for cartilage matrix with Alcian blue (pH 2.5) and counterstained with haematoxylin and eosin.

resorptions or dead fetuses were found and litter sizes were normal (data not shown), this distribution is striking in view of the expected frequency of mendelian segregation. To investigate the cause of this transmission distortion we have mated heterozygous males and females to wild-type mice. There were 51% of heterozygotes from male backcrosses, whereas only 34% of heterozygotes from female backcrosses were recovered (Table 1), implying a distorted female transmission frequency. Homozygous males and females are both fertile, although sexual maturation apparently occurs later than in wild-type mice (~10–15 weeks versus 4–5 weeks).

Mice lacking functional Fos exhibit no phenotypic changes within the first 10 days after birth. Thereafter, they could easily be distinguished from their littermates: (1) They are much smaller and their body weight is ~40–60% reduced (data not shown). The X-ray analysis in Fig. 2A shows that the mutant mice have shorter limbs and a shorter snout. (2) Mutant mice lack teeth. (3) The epiphyses and metaphyses of the long bones were much broader and the bone marrow spaces appeared to be calcified (Fig. 2A).

The predominant phenotype that develops in *fos* $-/-$ mice is a form of osteopetrosis. Figure 2B shows that the bone marrow spaces in the long bones of mutant mice are occupied by abundant bone and cartilage trabeculae, reducing the effective bone marrow cavity by ~80%. In addition, the cortical bone is extremely thin and underdeveloped, and there is an absence of osteoblastic cells along both the periosteal and the endosteal cortical surfaces (Fig. 2B). Multinucleated cells resembling

TABLE 1 Genotypes of offspring from heterozygote crosses

	+/+	+/-	-/-	Total
$F_1 \times F_1$:				
2-week-old mice	155 (40%)	186 (49%)	42 (11%)	383
Fetuses (E 12.5–16.5)	10 (19%)	35 (65%)	9 (16%)	54
Total	165 (38%)	221 (50%)	51 (12%)	437
$F_1(f) \times +/+ (m)$:	55 (66%)	29 (34%)	—	84
$+/(f) \times F_1 (m)$:	47 (49%)	50 (51%)	—	97

Analysis of *c-fos* genotypes using Southern blots in offspring generated from heterozygote intercrosses ($F_1 \times F_1$) and backcrosses ($F_1 \times +/+$). Note the distortion observed in female backcrosses ($F_1(f) \times +/+ (m)$). +/+, Wild type; +/-, heterozygotes; -/-, homozygotes; f, female; m, male.

osteoclasts line some of these trabeculae (data not shown). Examination of the growth plates of homozygous animals showed that the zone of proliferating chondrocytes was greatly reduced and the longitudinal columns typical of the proliferation zone appeared highly irregular (Fig. 2B). There was also a corresponding increase in the zone of hypertrophic chondrocytes. The identical phenotype was observed in all other bones examined, for example, the bones of the vertebral column, tail and in the skull (data not shown). With regard to tooth development, histological analysis revealed that the incisors and molars were present but were obstructed by abnormal amounts of bone (Fig. 2C). Thus, odontogenesis is apparently not affected,

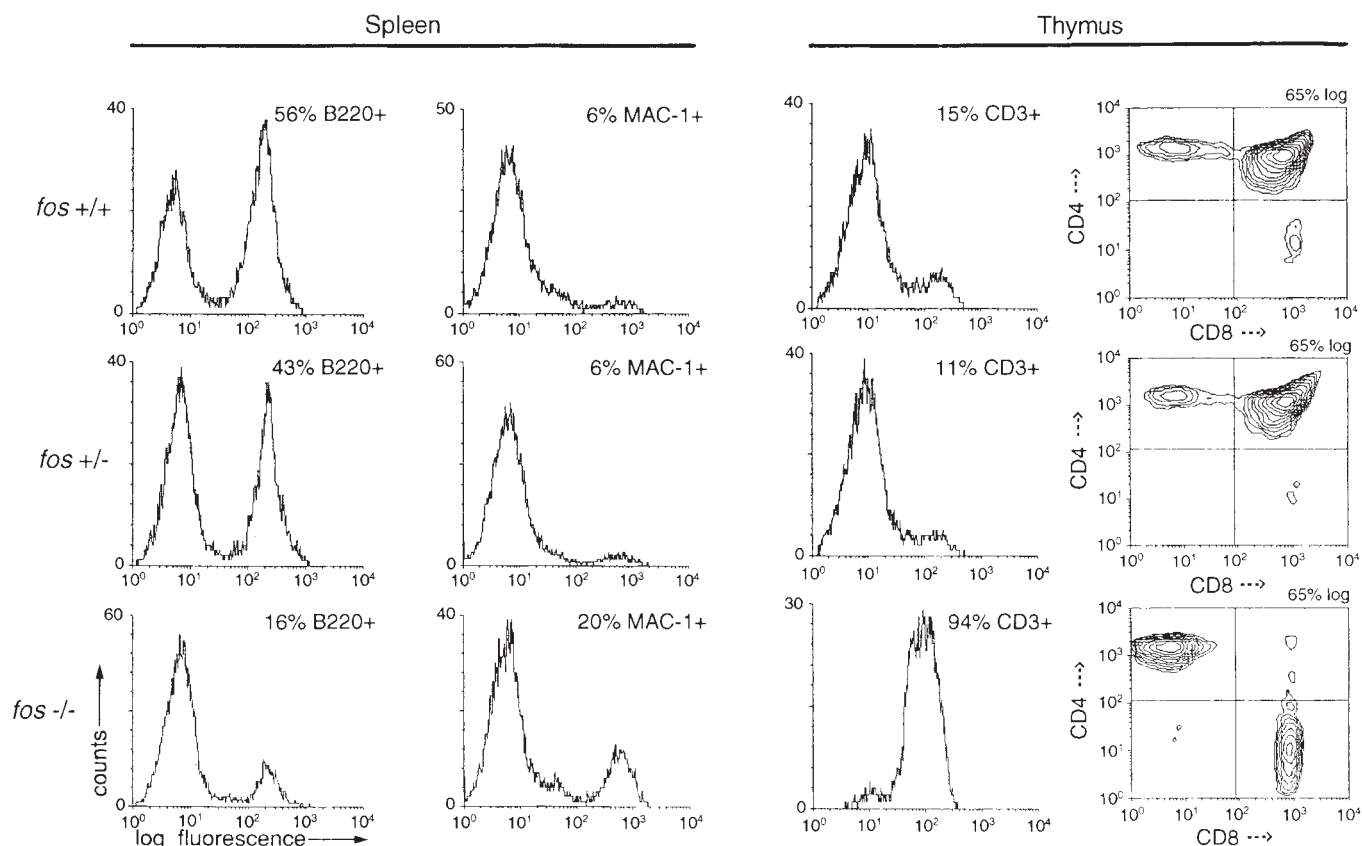


FIG. 3 Expression of surface markers on splenocytes and thymocytes isolated from *fos* $-/-$ mice and control littermates (*fos* $+/+$; *fos* $+/-$). Cells stained by fluorescent antibodies were analysed by flow cytometry. Data represent typical values from a representative mouse of each genotype. Two 6-week-old mice from each category have been analysed. METHODS. Cells (1×10^6) of spleen and thymus cell suspensions were stained with the following fluorescein-conjugated monoclonal antibodies: rat

anti-mouse B220 (RA3-6B2) and anti-mouse CD3 (145-2C-11). Staining with a rat anti-mouse Mac-1 (M1/70HL) monoclonal was followed by a fluorescein-conjugated antibody to rat immunoglobulin. Thymocytes were also double-stained with a phycoerythrin (PE)-conjugated rat anti-mouse CD4 monoclonal antibody (GK1.5/4) and a fluorescein-conjugated rat anti-mouse CD8 monoclonal antibody to (53-6.7). The data represent values for 1×10^4 viable cells.

and the failure of tooth eruption is probably secondary to the osteopetrosis. This osteopetrotic phenotype probably results from the failure of osteoclasts to resorb the bone and cartilage during development, as has also been observed in several natural osteopetrotic mutants and in mice lacking *c-src*²²⁻²⁵. Whether the defects in the *fos* mutants are due to impaired osteoclast function or altered bone marrow stromal environment is not clear. Finally, the bone defect suggests that osteogenesis and chondrogenesis appear to be affected directly, which correlates well with our observations in transgenic and chimaeric mice overexpressing *c-fos*¹⁶⁻¹⁸ (A.E.G. *et al.*, manuscript in preparation).

Autopsy of 4-6-week-old mutant mice revealed a reduction in the size of the thymus and no obvious differences in the spleen size. Histological analysis showed a less dense white pulp in mutant spleens and a poorly organized cortex and medulla in mutant thymuses (data not shown). The number of total splenocytes and thymocytes was reduced by 25% and 90%, respectively. Flow cytometry analysis using antibodies against cell-surface markers demonstrated a 75% reduction in the frequency of B220⁺ B cells in the spleen, whereas in the thymus the frequency of CD3⁺ T cells bearing CD4⁺ or CD8⁺ was increased six- to sevenfold. Furthermore, the frequency of double-positive CD4⁺/CD8⁺ immature thymocytes decreased by >90% (Fig. 3). In contrast, lymph nodes were unaltered with respect to size and the number of B220⁺ and CD3⁺ cells (data not shown). Two-week-old mutant mice have a normal thymus and an unaltered number of double-positive CD4⁺/CD8⁺ thymocytes, whereas B220⁺ cells are already reduced (data not shown). These results are consistent with the findings in transgenic mice overexpressing *c-fos* in haematopoietic tissues^{19,20}, but do not establish whether the lack of Fos affects B- and T-cell development directly or indirectly. With respect to the myeloid cells, mutant spleens contained a three- to fourfold increase in the frequency of Mac-1⁺ cells (Fig. 3) and the frequency of progenitor cells in spleen cell suspensions was also increased (data not shown). Analysis of peripheral blood of four 5-6-week-old mutant mice showed no significant differences in haematocrits and red blood cell counts. Thus, the reduced bone marrow space of the affected bones seems to be responsible for extramedullary haematopoiesis occurring in the spleen; we have not observed any haematopoietic activity in other organs, for example, in the liver.

It should be mentioned that subtle behavioural abnormalities have been observed in *fos* ^{-/-} mice in that they appear to be less responsive to external stimuli, possibly implying some deficiency in the central nervous system. Alternatively, these defects may arise as a consequence of the bone malformations in osteopetrosis²⁶. Histological analyses showed no gross abnormalities in the brain or in any other organs examined in the mutant mice.

This study shows that despite the common notion that Fos is a general regulator of cell proliferation, Fos has a specific function in bone, cartilage and haematopoietic cell development which cannot be substituted by other members of the AP-1 family. Our data demonstrate that, at least for *c-fos*, the endogenous expression profile is a valid indicator of specific function *in vivo*, and that useful information can be obtained from gain-of-function approaches. The *c-fos* mutant mice will provide an excellent tool to study AP-1-mediated signal transduction and oncogene cooperativity, as well as skeletogenesis and haematopoiesis. □

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Formation of haematopoietic microenvironment and haematopoietic stem cells from single human bone marrow stem cells

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HAEMATOPOIETIC stem cells are a population of cells capable both of self renewal and of differentiation into a variety of haematopoietic lineages¹⁻⁴. Enrichment techniques of human haematopoietic stem cells have used the expression of CD34, present on bone marrow progenitor cells⁵⁻⁷. But most CD34⁺ bone marrow cells are committed to their lineage, and more recent efforts have focused on the precise characterization of the pluripotent subset of CD34⁺ cells⁸⁻¹⁷. Here we report the characterization of two distinct subsets of pluripotent stem cells from human fetal bone marrow, a CD34⁺, HLA-DR⁺, CD38⁻ subset that can differentiate into all haematopoietic lineages, and a distinct more primitive subset, that is CD34⁺, HLA-DR⁻, CD38⁻, that can differentiate into haematopoietic precursors and stromal cells capable of supporting the differentiation of these precursors. These data represent, to our knowledge, the first identification of a single cell capable of reconstituting the haematopoietic cells and their associated bone marrow microenvironment.

We have shown that expression of CD38 on CD34⁺ human progenitor cells is correlated with lineage commitment of the progenitors and only the non-lineage committed CD34⁺ CD38⁻ cells were capable of extensive self renewal¹¹. We used HLA-DR to further subdivide these non-lineage committed cells. In three-colour immunofluorescence experiments, the expression of

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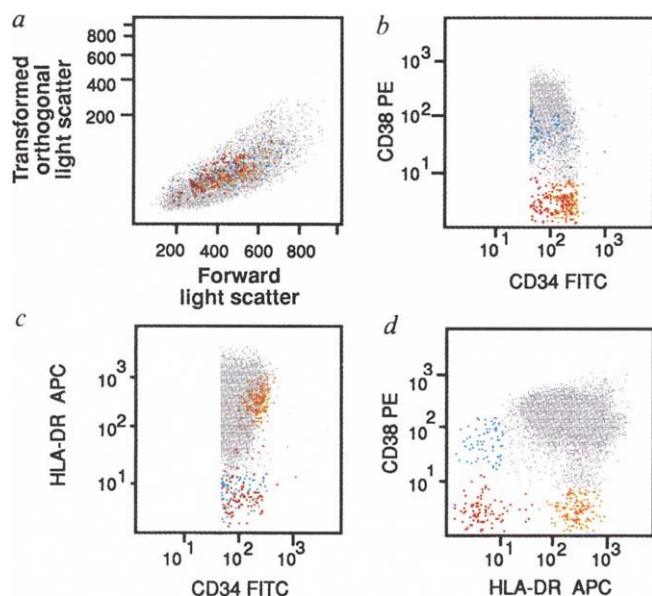


FIG. 1 Fetal bone marrows were from aborted fetuses (16–22 weeks gestational age) and used following the guidelines of the institutional review board of Stanford University Medical Center on the use of Human Subjects in Medical Research. Bone marrow cells were isolated by flushing intramedullary cavities of the femurs with RPMI 1640 medium with 10% FCS followed by density gradient sedimentation over Ficoll-Hypaque (Histopaque 1077, Sigma). The absolute number of cells recovered varied between $5\text{--}25 \times 10^6$. The cells were immunofluorescently labelled with CD34 (HPCA-2 (8G12) FITC), CD38 (Leu17 PE) and HLA-DR biotin followed by an incubation with streptavidin allophycocyanin (Becton Dickinson Immunocytometry Systems (BDIS)).

METHODS. Flow cytometric analysis was done on a FACStar Plus equipped with an Argon ion laser tuned at 488 nm and a Helium Neon laser (633nm) (BDIS). Data acquisition was done with the Lysys 2.0 (BDIS) with gates on light scattering and CD34⁺ cells. Forward light scattering, orthogonal light scattering and three fluorescence signals were determined for each cell and the 10,000 cells were stored in listmode data files. The analysis of the five-dimensional data was done with the PAINT-A-Gate^{plus} software (BDIS). The CD34⁺, HLA-DR⁻, CD38⁻ are coloured red; the CD34⁺, HLA-DR⁺, CD38⁻ yellow, the CD34⁺, HLA-DR⁻, CD38⁺ blue and the CD34⁺, HLA-DR⁺, CD38⁺ grey.

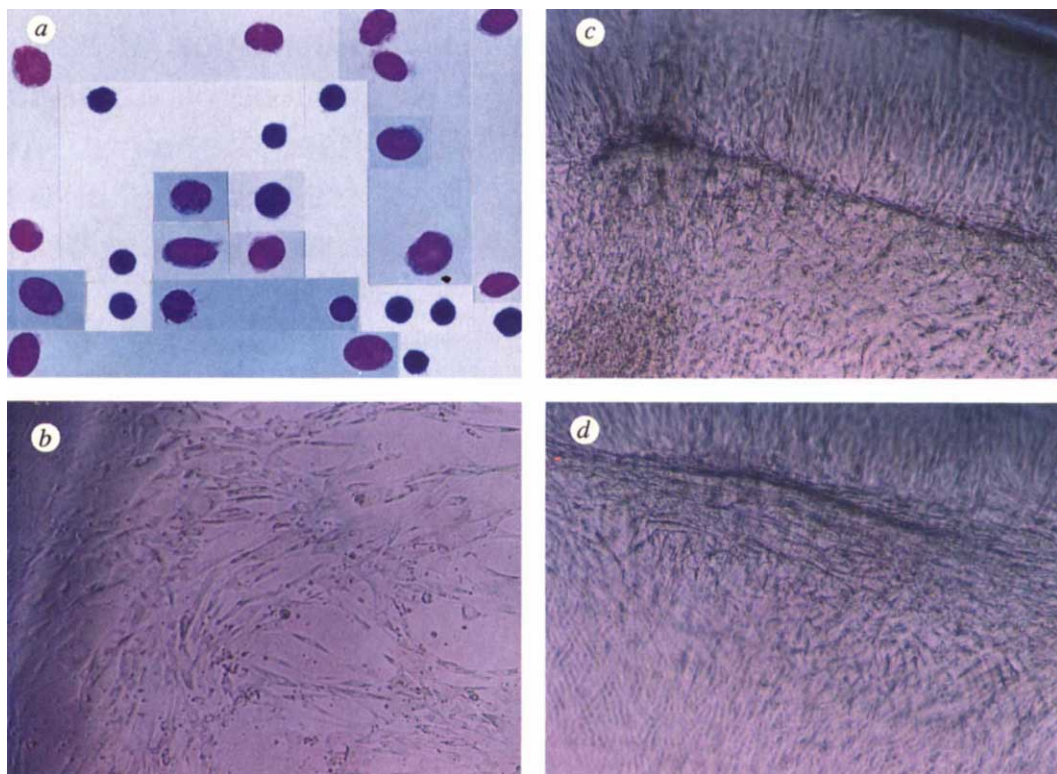
FIG. 2 a, Composition of photomicrographs of Wright-Giemsa stained cells from sorted CD34⁺, HLA-DR⁻, CD38⁻ cells (coloured red in Fig. 1).

To prevent cell loss no regular cytospin preparations were made, but cells were directly sorted onto a slide covered with CellTak (Collaborative, Bedford, MA) on top of which 100 μ l RPMI with 10% FCS was placed. After 500 cells were sorted, the slide was incubated for 10 min at 37 °C in a fully humidified incubator, then centrifuged for 20 min at 100g and stained with Wright-Giemsa. The dark blue coloured cells with irregular shaped nucleus are similar to the CD34⁺, HLA-DR⁺, CD38⁻ cells and the CD34⁺, CD38⁻ cells in adult bone marrow¹¹. The purple coloured cells resemble primitive mesenchymal elements of which the cells with the oval shaped nucleus and fine nucleoli could represent stromal precursors.

b. Photomicrograph taken from a well after 9 days of culture of a single CD34⁺, HLA-DR⁻, CD38⁻ cell (magnification $\times 200$). The majority of the cells can be identified as fibroblasts which are randomly distributed.

c. Photomicrograph taken from a well after 12 days of culture of a single CD34⁺, HLA-DR⁻, CD38⁻ cell (magnification $\times 200$). A variety of stromal components can be identified, but now the cells are organized on two sites of a longitudinal array of cells with deposits of extracellular matrix.

d. Photomicrograph taken from a well after 15 days of culture of a single CD34⁺, HLA-DR⁻, CD38⁻ cell (magnification $\times 200$). The cells now have formed a structure surrounded by stromal cells, arranged orthogonal on the structure.



METHODS. Cell sorting was done on a FACStar Plus (BDIS) using the Automated Cell Deposition Unit. The cells coloured red, yellow, blue and grey in Fig. 1 were sorted singly into 96-well flat-bottom plates. To eliminate doublets and cell aggregates a pulse processor module was used on the forward light scatter parameter, in addition cells with large orthogonal and forward light scatter were excluded. The accuracy of the cell deposition unit was tested by sorting single 6 μ m yellow-green fluorescent beads (Fluoresbrite, Polysciences Warrington, PA) into twelve 96-well flat-bottom plates. Examination of the plates with a fluorescent microscope showed that in only 1 of the 1,152 examined wells two beads were found, in 7 of the 1,152 wells no beads were found whereas in the remaining 1,144 wells 1 bead was found. In the liquid culture system each well contained a 200 μ l mixture of alpha medium, 12.5% horse serum 12.5% FCS, 10^{-4} M 2-mercaptoethanol, 2 mM L-glutamine, 0.2 nM i-inositol, 20 μ M folic acid, antibiotics, 2.5 ng/ml b-FGF, 10 ng/ml IGF-1 (Collaborative Bedford, MA). All cultures were incubated in 5% CO₂ in air at 37 °C in a fully humidified incubator.

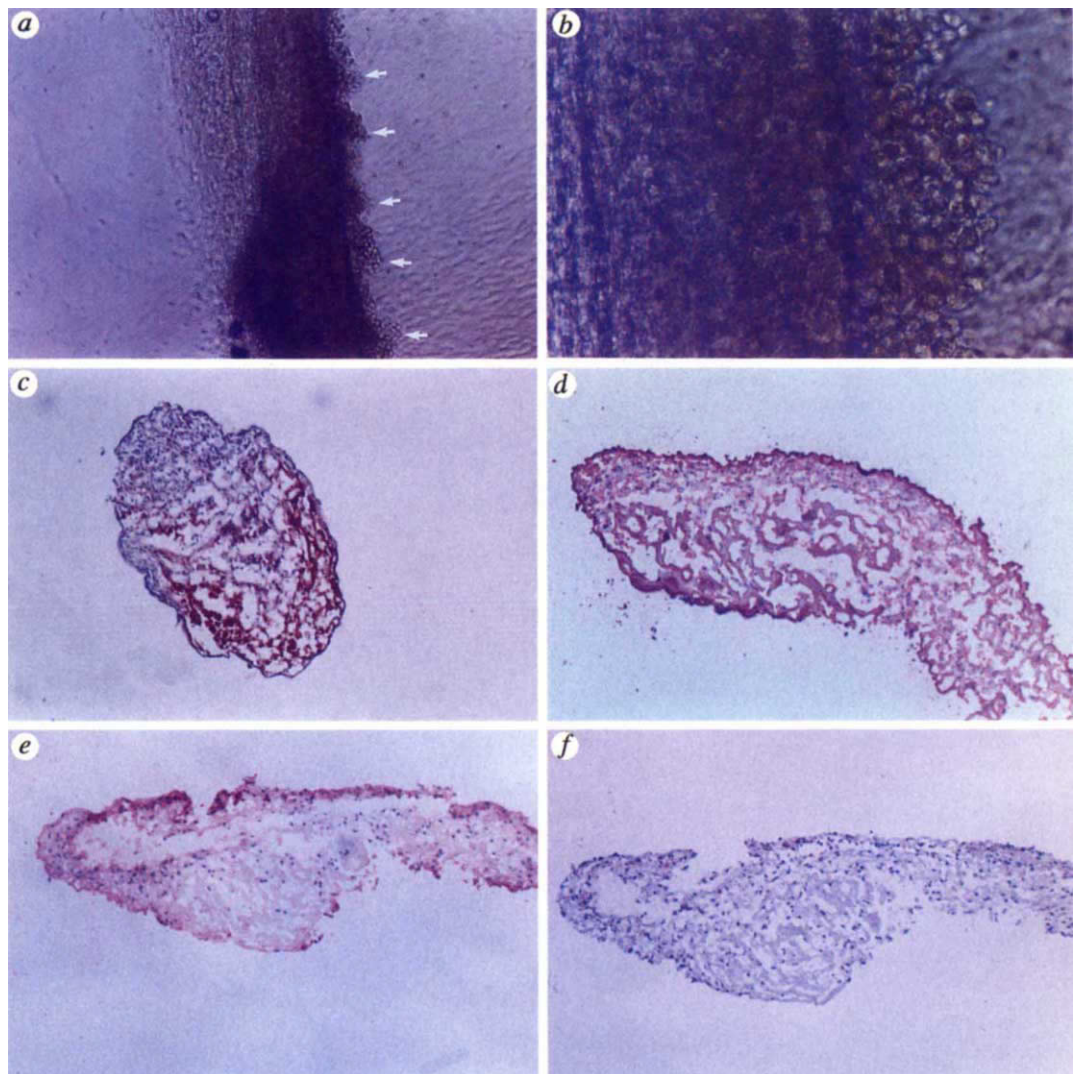
CD38 and HLA-DR was assessed on CD34⁺ cells in eight samples of human fetal bone marrow (gestational age ranged from 16 to 22 weeks). The frequency of the CD34⁺ cells in the fetal bone marrows was 8% (s.d. ± 3). Within the CD34⁺ cells four populations were identified: (1) HLA-DR⁻, CD38⁻ (4%, s.d. ± 3); (2) HLA-DR⁺, CD38⁻ (4%, s.d. ± 2); (3) HLA-DR⁻, CD38⁺ (3%, s.d. ± 2); and (4) HLA-DR⁺, CD38⁺ (89%, s.d. ± 5). Figure 1 illustrates a typical flow cytometric analysis of CD34⁺ fetal bone marrow cells; the CD34⁺, HLA-DR⁻, CD38⁻ cells were coloured red, the CD34⁺, HLA-DR⁺, CD38⁻ cells were coloured yellow; the CD34⁺, HLA-DR⁻, CD38⁺ cells blue; and the CD34⁺, HLA-DR⁺, CD38⁺ cells grey. Consistent with previous experiments, phenotyping and cell sorting studies revealed that the CD38⁺ cell populations were heterogeneous in morphology containing myeloblasts, erythroblasts, lymphoblasts as well as megakaryoblasts and expressed lineage-associated antigens. The HLA-DR⁺, CD38⁻ cells were homogeneous, primitive blast cells by morphology. In contrast, primitive blast elements were admixed with primitive mesenchymal elements in the HLA-DR⁻, CD38⁻ cell population (Fig. 2a).

The four CD34⁺ cell fractions based on HLA-DR and CD38 expression (coloured red, yellow, blue and grey in Fig. 1), were sorted singly in 96-well plates and assayed for their ability to form colonies in alpha medium (containing interleukin-3 (IL-3),

interleukin 6 (IL-6), stem cell factor (SCF) granulocyte macrophage colony-stimulating factor (GM-CSF), erythropoietin (Epo), insulin-like growth factor-1 (IGF-1), basic-fibroblast growth factor (b-FGF), 12.5% horse serum and 12.5% fetal calf serum (FCS). In eight experiments an average of 6% (range 0–13%) of the HLA-DR⁻, CD38⁻ cells, 50% (range 21–76%) of the HLA-DR⁺, CD38⁻ cells, 35% (range 24–39%) of the HLA-DR⁻, CD38⁺ cells and 16% (range 9–35%) of the HLA-DR⁺, CD38⁺ cells gave rise to haematopoietic colonies 14 days after cell sorting. In control experiments in which cells were sorted into the medium with 12.5% horse serum and 12.5% FCS no colony formation was found. Replating of the formed colonies confirmed our previous results in that only the colonies originating from the CD34⁺, CD38⁻ subsets could reform colonies after repetitive replating¹¹.

The presence of primitive mesenchymal elements in the HLA-DR⁻, CD38⁻ cell population suggested that this subset might enclose a subset of stem cells that could give rise to both the bone marrow microenvironment and the haematopoietic elements. We thus probed for the presence of stem cells that were not dependent on haematopoietic growth factors. To exclude stromal precursors that are potentially within the CD34⁺, CD38⁻, HLA-DR⁻ cell-population gates were used on the low-light scatter region^{16,17}. The four CD34⁺ cell fractions based on

FIG. 3 a, Photomicrograph taken from a well after 17 days of culture of a single CD34⁺, HLA-DR⁻, CD38⁻ cell (magnification $\times 200$). Note the presence of a bone/cartilage-like structure with longitudinally arranged cell arrays surrounded by stromal cells. Colonies of cells are present between the structure and the surrounding stromal cells (indicated with arrows). b, The same structure but now with a $\times 400$ magnification. c, Photomicrograph with a $\times 100$ magnification of the alkaline phosphatase enzyme activity of a frozen section of a structure, the section was counter-stained with Hematoxyline (Sigma). d, e, f, Photomicrographs with a $\times 100$ magnification of frozen sections from one structure stained with chondroitin sulphate, Stro-1 and cytokeratin, respectively. Antibody-staining was visualized by development with alkaline phosphate substrate after endogenous alkaline phosphate was blocked (Fast Red, Biomed, Foster City, CA). The sections were counter-stained with Hematoxyline. (Antibodies against chondroitin sulphate, fibroblast surface protein, collagen IV, laminin, fibronectin, elastin, smooth muscle actin and vimentin were obtained from Sigma, Stro-1 from the Developmental Studies Hybridoma Bank, Iowa City, cytokeratin from BDIS and factor VIII from ZYMED laboratories, South San-Francisco, CA).



HLA-DR and CD38 expression (coloured red, yellow, blue and grey in Fig. 1), were sorted singly in 96-well plates in alpha medium containing only IGF-1, b-FGF, 12.5% horse serum and 12.5% FCS. Only in the CD34⁺, HLA-DR⁻, CD38⁻ cell population single cells were found which produced randomly distributed ellipsoid shaped cells of which the majority could be identified as fibroblasts after 7–10 days of cell culture (Fig. 2b). Between 10–13 days of culture spindle cells appeared in these wells which gradually formed organized cell structures with longitudinally arranged cellular arrays (Fig. 2c). At day 15 to 17 complex composite structures of stromal elements emerged from a background of fibroblast-like spindle cells (Fig. 2d). At the border between these structures and the spindle cells haematopoietic cells appeared. Figure 3a, b illustrates such a structure obtained after 17 days of cell culture of a single CD34⁺, HLA-DR⁻, CD38⁻ cell. The position of the cell colonies is indicated with arrows in Fig. 3a and shown with a higher magnification in Fig. 3b. Morphologic light/electron microscopic histochemical and immunohistochemical analysis revealed that the stromal cell structures in these cultures contained primitive mesenchymal cells showing apparent differentiation along a variety of mesenchymal cell lineages, including fibroblast, adipocytes, smooth muscle cells, endothelial cells and chondrocytes/osteoblasts. Figure 3c shows the alkaline phosphatase enzyme activity of a structure and Fig. 3d shows extensive chondroitin sulphate immunoreactivity consistent with osteoblast and chondroblast differentiation. Within the structures, reactivity with the stromal cell antigen Stro-1 (Fig. 3e) as well as reactivity with factor VIII, fibroblast surface protein, collagen IV, laminin, vimentin, fibronectin, elastin and smooth muscle actin was found (not shown). No epithelial cell differentiation was identified as evident by the lack of reactivity with antibodies against cytokeratins (Fig. 3f).

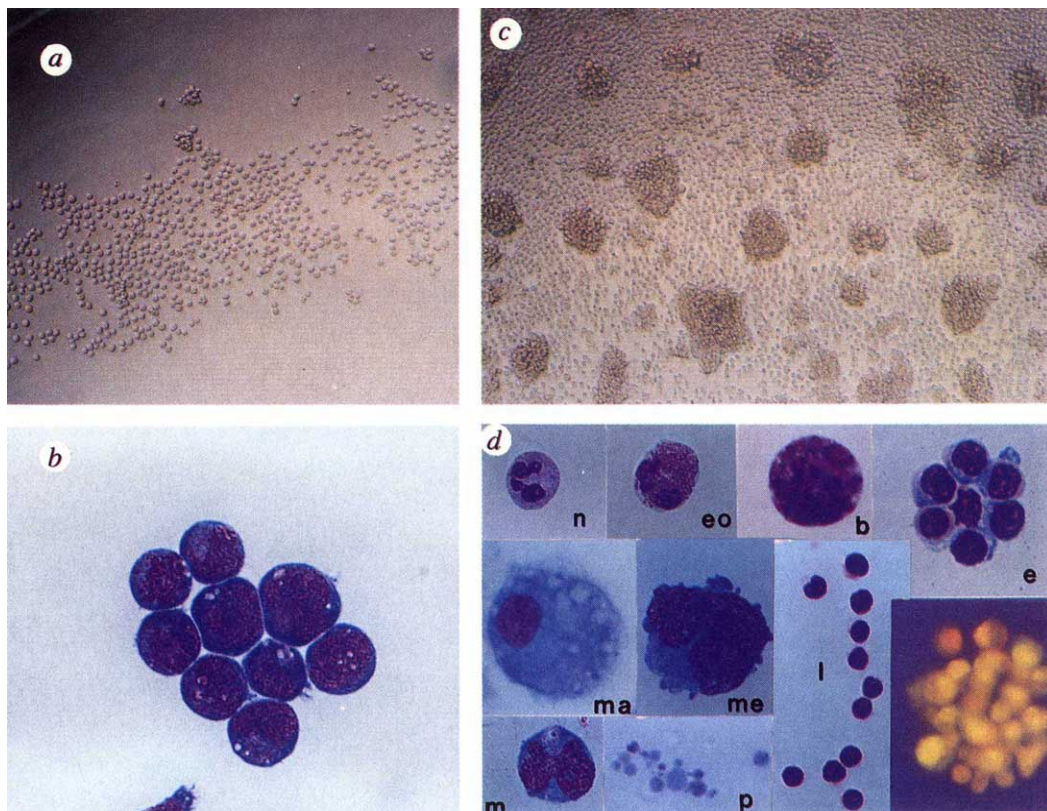
To verify the haematopoietic origin of the colonies indicated

with arrows in Fig. 3a, the colonies were selected, dispersed, and replated in alpha medium containing horse serum, FCS, IL-3, IL-6, GM-CSF, b-FGF, IGF-1, Epo and SCF. Two weeks after replating, typical haematopoietic blast colonies could be found (Fig. 4a, b). Parts of the colonies were used for morphologic examination or to probe for cell surface antigen expression, whereas the remaining cells were dispersed and replated. The replaced cells were cultured in identical media and were evaluated for colony formation after 2 weeks of culture. In 50% of the wells, second-generation haematopoietic colonies were found with varying morphologic appearance. A typical example is shown in Fig. 4c. The second-generation colonies were processed similarly to the first generation colonies and gave rise to third-generation colonies and these could also give rise to fourth-generation colonies. Morphology of the colonies showed that the percentage of blasts within the colonies decreased gradually from the first (68% blasts) to the second (41% blasts), third (14% blasts) and fourth (0% blasts) generations. The outcome of the lineage commitment of the colonies was not predictable and followed a random pattern. Neutrophils, eosinophils, basophils, erythrocytes, monocytes, macrophages, megakaryocytes, platelets and B-lymphocytes could be found either in the first, second, third or fourth generations (Fig. 4d). Identity of cells which appeared as lymphoblasts by morphology was confirmed by immunofluorescence cell surface staining with either CD10 fluorescein isothiocyanate (FITC) or CD19 PE (Fig. 4d). No cells were found that were differentiated into the T lymphocyte lineage (using CD7 and CD3). These data indicate that the cells depicted from the structure illustrated in Fig. 3a, b do have properties expected from the haematopoietic stem cells, extensive self-renewal capacity and ability to differentiate into each of the haematopoietic cell lineages.

The frequency of single CD34⁺, HLA-DR⁻, CD38⁻ cells sorted into 96-well plates which gave rise to both a

FIG. 4 a, Photomicrograph of a typical blast colony taken from a well after 14 days of culture of cells depicted and dispersed from a colony shown in Fig. 3 (magnification $\times 200$). b, Photomicrograph of Wright-Giemsa-stained blast colony cells (magnification $\times 1,000$). c, Photomicrograph of second-generation colonies taken from a well after 14 days of culture of a dispersed and replated blast colony (magnification $\times 200$). d, Composition of photomicrographs of Wright-Giemsa-stained cells generated from colonies originated from replated blast colonies (magnification $\times 1,000$). Neutrophil, n; eosinophil, eo; basophil, b; erythrocytes, e; macrophage, ma; monocyte, m; megakaryocyte, me; platelets, p; and lymphocytes, l. The lower right corner shows a lymphoid colony immunofluorescently stained with CD10 FITC.

METHODS. In the liquid culture system each well contained the medium described in Fig. 2 and 10 ng ml⁻¹ rhIL-3, 500 U ml⁻¹ rhIL-6, 10 ng ml⁻¹ rhGM-CSF (Collaborative, Bedford, MA), 2.5 U ml⁻¹ rh Epo (AMGEN, Thousand Oaks, CA) and 50 ng ml⁻¹ human stem cell factor (SCF, Genzyme). The plates were observed between days 7 and 14 for the appearance of colonies. Replating of colonies was done by



dispersion of the individual colonies into 96-well flat-bottom plates containing the culture medium. Repetitive replating of colonies was done under identical conditions.

haematopoietic microenvironment and haematopoiesis in four experiments was 1%, 4%, 5% and 2%, respectively. In two experiments in which only single CD34⁺, HLA-DR⁻, CD38⁻ cells with relatively large forward light scatter were sorted no cell growth was observed, whereas in two other experiments single CD34⁺, HLA-DR⁻, CD38⁻ cells with low forward light scatter, 5% and 12%, respectively, of the single cells gave rise to both a haematopoietic microenvironment and haematopoiesis. No cells were found that gave rise to only haematopoietic colonies or only a haematopoietic microenvironment.

Self renewal ability of the formed structures was studied by disrupting and removing the structures and the supernatant, and replenishing of the media with serum, IGF-1 and b-FGF. After 2 to 5 days of culture only degenerated cells and debris could be found; however, the identical differentiation process developed as for the originally sorted single cells. After 15 to 17 days of culture, similar structures appeared including the presence of haematopoietic cell colonies. The disruption of the microstructure could be repeated and both the structure and the colonies reappeared in an identical timely fashion. These results provide direct evidence for the existence of a single class of common stem cells which can sequentially differentiate into both the microenvironment and the haematopoietic stem cells of human bone marrow. The observation that the structures repeatedly reconstructed themselves after disruption and supplementation with the media, IGF-1 and b-FGF, strongly suggests that the common stem cell of the haematopoietic cells and the haematopoietic microenvironment possesses the ability to self-renew. The data indicate that in early development of

the haematopoietic system, common stem cells first generate stromal stem cells which give rise to a haematopoietic microenvironment which then induce the common stem cells to differentiate into the haematopoietic stem cells.

The identification of common stem cells of both the haematopoietic microenvironment and the haematopoietic stem cells offers exciting new possibilities to study the haematopoietic system. For example, purified common stem cells might be the ideal target for gene therapy. Moreover, the involvement of these unique cells in malignancies of the haematopoietic system and its microenvironment can now be explored. □

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Reversal of pathology in murine mucopolysaccharidosis type VII by somatic cell gene transfer

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AN inherited deficiency of β -glucuronidase in humans¹, mice² and dogs³ causes mucopolysaccharidosis VII (Sly syndrome), a progressive degenerative disease that reduces lifespan (to an average of 5 months in mice²) and results from lysosomal storage of undegraded glycosaminoglycans in the spleen, liver, kidney, cornea, brain and skeletal system^{1–4}. Bone marrow transplantation in mutant mice provides a source of normal enzyme ('cross-correction'⁵), which substantially improves the clinical condition and extends the average lifespan to 18 months⁶. Gene therapy by transfer of a β -glucuronidase gene into mutant haematopoietic stem cells is an alternative approach^{7,8}, but it is not known whether the low expression of vector-transferred genes *in vivo*^{9,10} would be sufficiently effective. Here we show that retroviral vector-mediated transfer of the gene to mutant stem cells results in long-term expression of low levels of β -glucuronidase which partially corrects the disease by reducing lysosomal storage in liver and spleen.

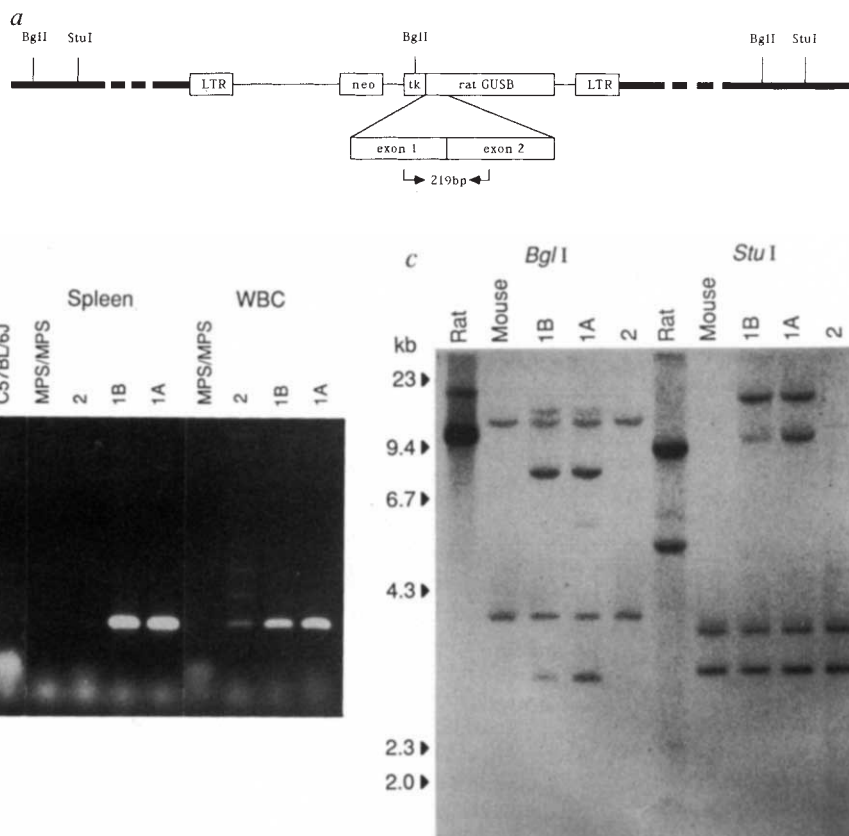
Bone marrow cells from mucopolysaccharidosis (MPS) VII mice were infected with the NTK-BGEO vector^{7,8,11,12} (Fig. 1a) and transplanted intravenously into eight lethally irradiated adult MPS VII recipients. Spleens from two mice examined 11 days after transplantation contained β -glucuronidase-positive

cells^{6,7,12,13}. One of four animals alive 42 days after transplantation had detectable β -glucuronidase activity in circulating white blood cells. To demonstrate that long-term repopulating stem cells in this mouse contained vector proviruses, we pooled spleen and bone marrow cells and transplanted them into irradiated adult MPS VII mice. The two secondary recipients (mice 1A and 1B) and one of the three remaining primary recipients (mouse 2) survived longer than six months after transplantation, which indicated that they had been reconstituted by stem cells, and were killed for further evaluation.

Vector proviral sequences were detected in all three animals by polymerase chain reaction (PCR) (Fig. 1b), indicating that vector-infected stem cells had reconstituted the haematopoietic system of each mouse⁸. Analysis of vector provirus integration patterns suggested that the repopulating cells were derived predominantly from a small number of transduced stem cells¹⁴ (Fig. 1c). The secondary recipients (1A and 1B), which appeared to have the same viral integration sites, were probably reconstituted by cells derived from common stem cell precursors in the primary donor¹⁵. The strength of PCR and hybridization signals suggest that a substantial proportion of the repopulating cells contained vector provirus.

The level of β -glucuronidase expression was assayed in various organs, including liver, spleen and bone marrow (Fig. 2a). Mouse 1A had 6% of normal activity in spleen and lymph nodes, 2% in thymus and liver, 26% in bone marrow, and 1% or less in lung and kidney. Mouse 1B had 1–2% of normal activity in bone marrow and spleen, 9% in thymus, and less than 1% in lung and liver. In mouse 2, β -glucuronidase activity was only found in bone marrow and spleen and was less than 1% of normal. α -Galactosidase and β -hexosaminidase activities were also measured as they are increased in untreated MPS VII mice² and correction of the rise after treatment by bone marrow transplantation is correlated with reduction of storage⁶. Mice 1A and 1B had reduced levels of α -galactosidase in liver, spleen, and marrow (Fig. 2b), and of β -hexosaminidase in liver and marrow (Fig. 2c). No changes were apparent in mouse 2 (Fig. 2b and c).

FIG. 1 Vector infection, cell transplantation, and detection of vector proviral sequences in treated mice. **a**, Map of the β -glucuronidase (GUSB)-expression vector⁷ showing restriction enzyme sites and location of oligonucleotide primers used for the PCR. The vector virus, which expresses normal levels of GUSB and restores substrate degradation to normal *in vitro*^{7,11}, was produced in GP + E86 packaging cells¹⁹ at $\sim 10^6$ PFU per ml (G418 resistance). Stem cells were enriched from MPS VII bone marrow by density gradient centrifugation^{8,20}, cultured in 200 U ml^{-1} each of recombinant mouse interleukin-3 and recombinant human interleukin-6 (GIBCO/BRL) for 96 h at 37°C and 5% CO_2 (refs 8, 21). During the final 48 h, cells were co-cultured with the packaging cells in $8 \mu\text{g ml}^{-1}$ polybrene^{8,21}. 4×10^5 cells were injected intravenously into 8 irradiated (9 Gy) MPS VII recipients (32–52 days old)^{6,8}. Two mice were analysed for GUSB expression at 11 days post-transplant. Two mice that died between 11 and 42 days and 2 that died between 42 and 180 days post-transplant were not analysed. At 42 days post-transplant, bone marrow and spleen cells were isolated from a mouse with GUSB-positive cells in blood and spleen, and 1.7% normal activity in liver. 1.4×10^7 pooled spleen and bone marrow cells from this animal were injected intravenously into 2 irradiated (9 Gy) secondary recipients (mice 1A and 1B) (56 days old). **b**, Detection by PCR of proviral sequences in the DNA of spleen and white blood cells (WBC) of treated mice (1A, 1B and 2) at 6 months post-transplantation (221, 221 and 288 days old, respectively). The first lane contains the PCR product amplified from a plasmid containing the rat GUSB cDNA²²; the second, third and seventh lanes show the results of amplifying genomic DNA from normal and untreated MPS VII mice. The reaction was done using previously described methods and oligonucleotides⁸. **c**, Southern blot of spleen DNA hybridized with the rat GUSB cDNA probe (89% nucleic acid identity with mouse GUSB). *Bgl*II cuts the vector in the thymidine kinase promoter, so the bands represent the 3' end of the vector and flanking genomic sequences. Two bands (about 4 and 12 kb) of the mouse GUSB gene hybridized with the rat probe. Four unique provirus integration fragments were seen as bands of ~ 3.5 , 5, 8 and 15 kb in animals 1A and 1B



(the 5-kb band can be seen in the original autoradiogram). *Stu*I does not cut within the vector, therefore the bands include both 5' and 3' flanking DNA and the hybridizing fragments were expected to be much larger than the 7 kb of the vector⁷. Two bands of genomic GUSB were seen at ~ 3 and 4 kb. Unique bands of ~ 10 kb were present in animals 1A and 1B. The bands from animals 1A and 1B that migrate at >20 kb probably included multiple provirus-containing fragments that were not resolved on the gel. No unique fragments were identified in mouse 2, but proviral sequences were present by PCR (see **b**). As the PCR signal and GUSB activity were low in this animal, there were probably too few cells from unique clones to produce a detectable signal on the Southern blot. The DNA was prepared, digested, electrophoresed on a 0.8% agarose gel, blotted and probed as described^{2,8}.

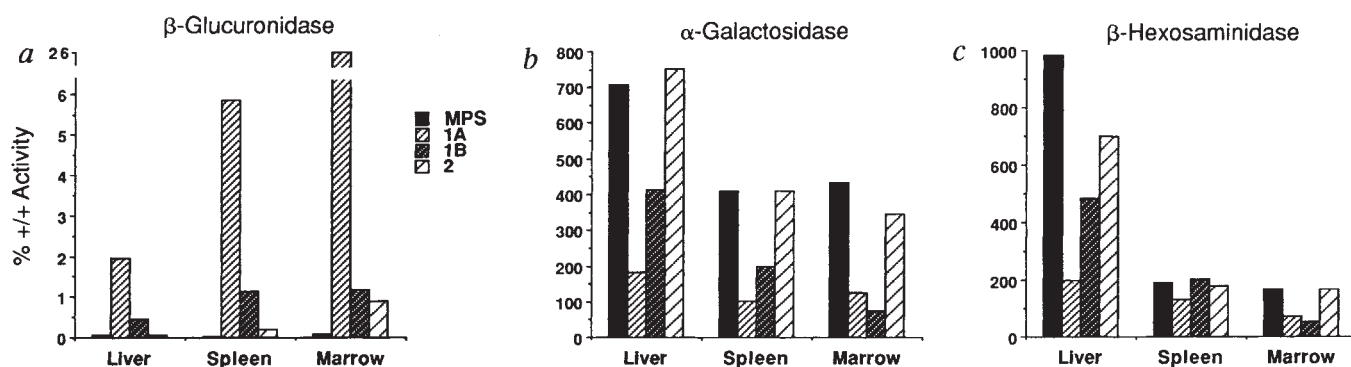


FIG. 2 Lysosomal enzyme activity in tissues of three treated mice and an untreated MPS VII control expressed as a percentage of the activity in

normal mice. The specific activity (nmol substrate cleaved per hour per mg protein) was determined on duplicate samples as described^{2,6}.

Localization of β -glucuronidase activity in tissues^{6,13} (Fig. 3) showed positive cells distributed throughout the liver and spleen in mice 1A and 1B (Fig. 3c, d, h, i). In liver, the activity was seen in sinusoidal lining cells (presumably Kupffer cells) and was not visible in hepatocytes. In mouse 2, a few small clusters of positive cells were seen in liver (Fig. 3e) but most of the tissue was negative. These results suggest that only a subset of

the donor cells were expressing the transferred gene, consistent with downregulation of vector expression^{9,10} or the presence of uninfected donor cells.

The characteristic histopathological feature of MPS VII is the presence of large cytoplasmic vacuoles, representing lysosomes distended by accumulated undegraded glycosaminoglycans (GAGs)^{1-6,11,16}. The reduction of lysosomal storage observed in

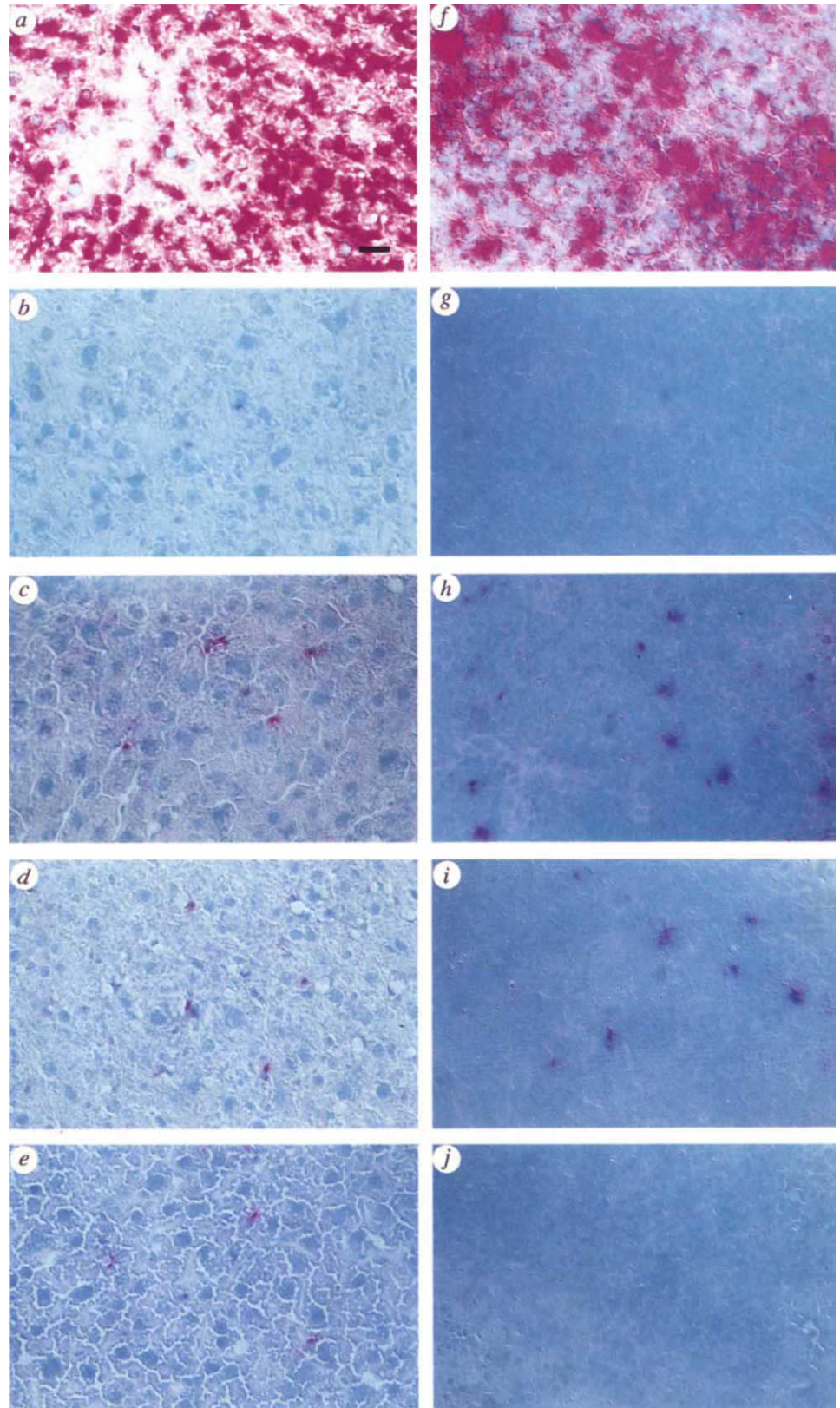


FIG. 3 Expression of GUSB enzymatic activity in tissues of MPS VII mice treated by gene transfer. The positive cells stain bright red^{6,7,12,13}. a-e, Liver; f-j, spleen; a, f, normal; b, g, untreated MPS VII; c, h, mouse 1A; d, i, mouse 1B; e, j, mouse 2. Scale bar in a represents 20 μ m. The tissues to be tested for GUSB activity were isolated, frozen, cut into 10- μ m sections, assayed for activity using a histochemical reaction^{6,12,13}, and photographed using differential interference microscopy¹³.

liver and spleen microscopically (Fig. 4) correlated with the levels of β -glucuronidase enzymatic activity (Figs 2 and 3). The liver and spleen of mouse 1A had 2% and 6% of the normal activity, respectively, and there was marked reduction in lysosomal storage (Fig. 4c, h). The hepatocytes were indistinguishable from normal by light microscopy (Fig. 4c) and electron microscopy (not shown). In mouse 1B the amount of storage

in liver and spleen cells was also reduced compared with untreated controls (Fig. 4d, i). In contrast, in mouse 2 only a few positive cells were seen in the *in situ* assay, very low or no activity was measurable by biochemical assay, and no reduction in storage was apparent histologically (Fig. 4e, j).

The dramatic decreases in lysosomal storage observed in liver and spleen demonstrate that low levels of β -glucuronidase in these tissues are sufficient to metabolize both the stored and newly produced GAGs. Hepatocytes had greatly reduced storage even though they appeared negative for β -glucuronidase activity histochemically (Fig. 3). Results were similar following normal bone marrow transplantation, when 15–18% of normal β -glucuronidase activity was present in liver but was detected in sinusoidal lining cells, not in hepatocytes⁶. Perhaps the *in situ* assay is not sufficiently sensitive to detect small amounts of cross-corrective enzyme in hepatocytes. Alternatively, proteoglycan turnover in the liver may be primarily a Kupffer cell function and MPS VII hepatocytes only store GAGs when Kupffer cells are metabolically blocked. Accordingly, vector correction of MPS VII Kupffer cells might restore the preferred pathway of proteoglycan turnover, and substrate stored in hepatocytes may decrease by nonspecific release of undegraded GAGs resulting from normal turnover of the endosome-lysosome compartment.

The therapeutic response is clearly dependent on the level of β -glucuronidase expression in the diseased mice. Here, gene transfer to haematopoietic stem cells resulted in the expression of low levels of β -glucuronidase and clearance of storage in liver and spleen, but not in kidney and cornea (data not shown). In contrast, the higher level of enzyme expressed after treatment by bone marrow transplantation reduces storage in all four tissues⁶. The pathology of the brain and skeleton is not corrected even by the higher levels of enzyme activity in recipients of normal marrow⁶, so different approaches¹³ will be necessary to effect a complete cure. Nevertheless, the degree of correction achieved by low-level expression of the normal enzyme appeared to improve the clinical well-being of the animals in that mice 1A and 1B were more active and looked healthier than age-matched (8 months) untreated MPS VII mice. Mice that responded to gene therapy appeared similar to long-term recipients of bone marrow transplants in which lifespan is extended⁶.

The discovery that low levels of expression of the transferred β -glucuronidase gene can strikingly reduce storage suggests new approaches for treatment of lysosomal storage diseases. Partial replacement of bone marrow with a small number of vector-corrected stem cells may be sufficient to correct the metabolic defects in liver and spleen without inducing the severe complications associated

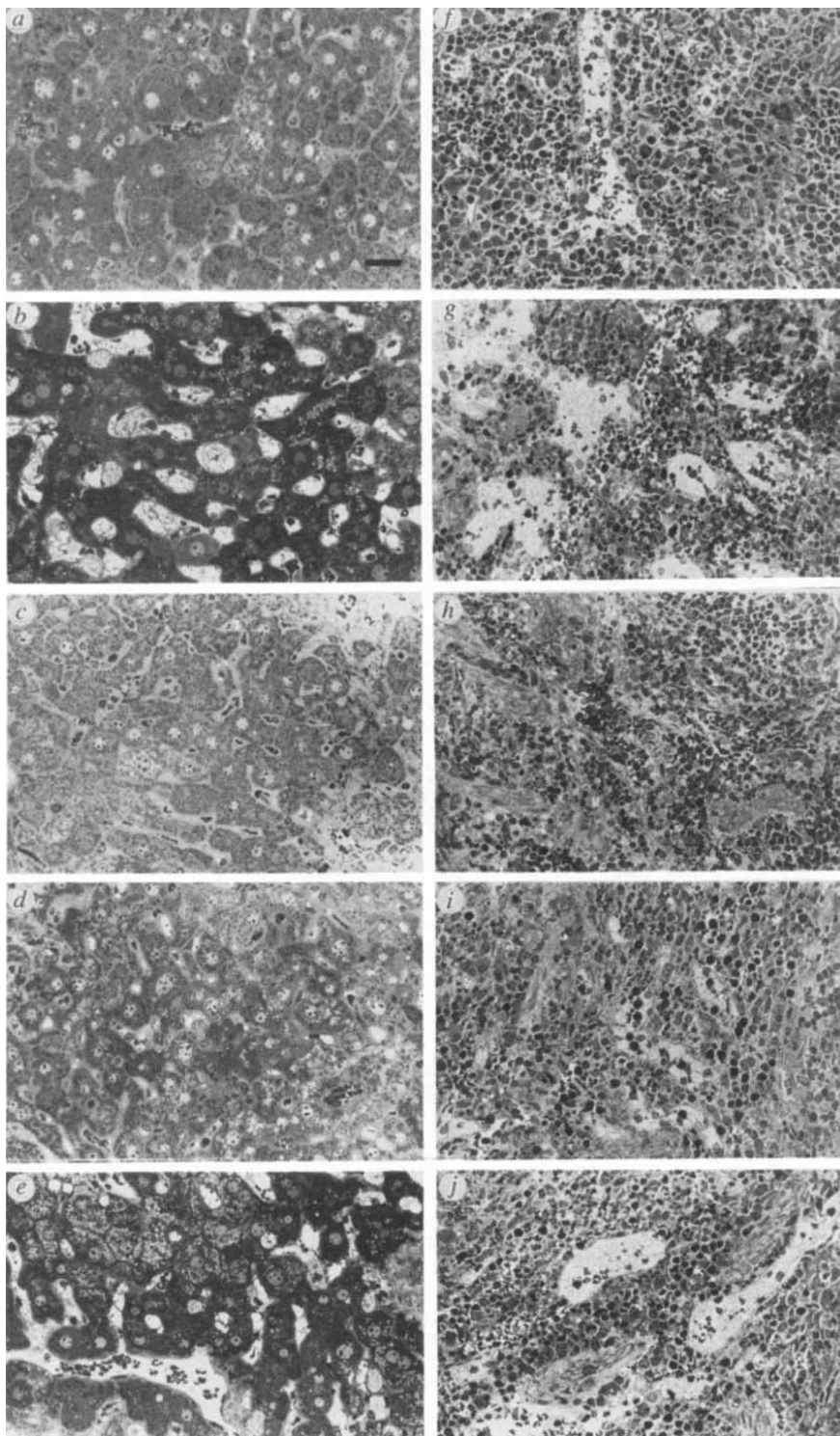


FIG. 4 Changes in lysosomal storage in liver and spleen of MPS VII mice after long-term haematopoietic reconstitution from vector-infected stem cells. Lysosomal storage is represented by the large cytoplasmic vacuoles. a–e, Liver; f–j, spleen; a, f, normal; b, g, untreated MPS VII; c, h, mouse 1A; d, i, mouse 1B; e, j, mouse 2. Scale bar in a represents 33 μ m. Tissues were fixed, embedded, sectioned (1 μ m), stained with toluidine blue and photographed as described^{2,6,16}.

with bone marrow ablation. It may now be feasible to deliver therapeutically effective amounts of normal enzyme to patients by gene transfer to tissues such as muscle¹², liver¹⁷ or organoids¹⁸, in addition to haematopoietic stem cells. Our results indicate that the amount of functional enzyme that can be delivered, even though it is much lower than normal, may still be of therapeutic value to patients with mucopolysaccharidoses. □

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Brain-derived neurotrophic factor rescues spinal motor neurons from axotomy-induced cell death

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CURRENT ideas about the dependence of neurons on target-derived growth factors were formulated on the basis of experiments involving neurons with projections to the periphery^{1,2}. Nerve growth factor (NGF) and recently identified members of the NGF family of neuronal growth factors, known as neurotrophins, are thought to regulate survival of sympathetic and certain populations of sensory ganglion cells during development^{3–8}. Far less is known about factors that regulate the survival of spinal and cranial motor neurons, which also project to peripheral targets. NGF has not been shown to influence motor neuron survival^{9,10}, and whether the newly identified neurotrophins promote motor neuron survival is unknown. We show here that brain-derived neurotrophic factor (BDNF) is retrogradely transported by motor neurons in neonatal rats and that local application of BDNF to transected sciatic nerve prevents the massive death of motor neurons that normally follows axotomy in the neonatal period. These results show that BDNF has survival-promoting effects on motor neurons *in vivo* and suggest that BDNF may influence motor neuron survival during development.

We first examined the ability of spinal motor neurons to retrogradely transport BDNF in neonatal rats. Recombinant human BDNF was produced and purified as described¹¹ and iodinated¹². ¹²⁵I-labelled BDNF was injected into the right lower

TABLE 1 Motor neuron counts in L4–5 spinal cord segments of 6-day-old rats after right sciatic nerve transection on postnatal day 1

Animal	Factor	No. of motor neurons		Survival (R/L × %)	Mean ± s.d.
		Right	Left		
1	BDNF	1,088	1,134	96	92 ± 6.6*
2	BDNF	922	1,098	84	
3	BDNF	1,065	1,216	88	
4	BDNF	1,117	1,142	98	
5	Cytochrome c	949	1,588	60	60 ± 5.7
6	Cytochrome c	855	1,298	66	
7	Cytochrome c	716	1,191	60	
8	Cytochrome c	531	1,022	52	

Motor neuron survival is presented as the percentage of counts on the right (R; lesioned side) over left (L; non-lesioned side).

*Significantly more motor neurons survived after treatment with BDNF than after treatment with cytochrome c ($P < 0.0002$, Student *t*-test, two tails).

leg of 2-day-old rats and retrograde transport was examined by emulsion autoradiography of lumbar spinal cord sections (Fig. 1). Dense accumulation of silver grains was found over motor neurons in the ipsilateral spinal motor column (Fig. 1a). No silver grains above background level were observed in the contralateral spinal cord. Co-injection of a 50-fold excess of unlabelled BDNF completely blocked transport of ¹²⁵I-labelled BDNF (Fig. 1b). ¹²⁵I-labelled cytochrome c, which has physical chemical properties that are similar to BDNF, was not retrogradely transported by motor neurons after injection into the leg (data not shown). These results show that the ability of motor neurons to transport BDNF in a specific receptor-mediated fashion, previously demonstrated in adult rats¹¹, is present by postnatal day 2.

The demonstration of specific retrograde transport of BDNF prompted us to ask whether this molecule has biological effects on spinal motor neurons. A rapid and reproducible motor neuron cell death occurs after sciatic nerve axotomy in neonatal rats^{13,14}. Motor neurons with axons in the sciatic nerve account for 40% of motor neurons in the lower lumbar spinal cord and virtually all are lesioned by axotomy in the first 48 h of life¹³. We therefore used this experimental paradigm to assess the protective effects of BDNF on spinal motor neurons. BDNF (at 1 mg ml⁻¹ in PBS, corresponding to a dose of 5 µg g⁻¹ body weight) was subcutaneously injected into the right hindleg of newborn rats (day 0) within 2–3 h after birth. On day 1, the right sciatic nerve was cut near the obturator tendon in the thigh and a 3 × 3 × 3 mm³ Gelfoam presoaked in BDNF (1 mg ml⁻¹ in PBS) was implanted next to the proximal sciatic nerve stump. In addition we injected 5 µl of the same BDNF solution into the lesion site daily during days 4–5 in order to supply additional BDNF to the transected motor axons. Control animals received cytochrome c in the same dosages administered in exactly the same fashion. Animals were killed on day 6. Motor neurons were counted on both sides of lumbar segments L4 and L5 as described previously¹⁰.

Results of this analysis are shown in Fig. 2 and Table 1. Figure 2 shows representative cross-sections of lumbar spinal cords from a control animal that received cytochrome c (Fig. 2a) and an animal that received BDNF (Fig. 2b). Spinal motor neurons on the non-lesioned side (left; open arrows) and on the side of the sciatic nerve section (right; solid arrows) are shown. The loss of motor neurons after sciatic nerve section in control animals treated with cytochrome c is apparent. In contrast, in BDNF-treated animals, most motor neurons survived axotomy over the 7-day period of the experiment. Cell counts from control and experimental animals showed that 40% of motor neurons were lost in cytochrome c-treated animals compared with only 8% loss in animals that received BDNF (Table 1). These data show that BDNF largely prevents the death of motor neurons

that normally occurs after axotomy at this age in rats. The ability of BDNF to prevent motor neuron death *in vivo* is not shared by all neurotrophins. Application of NGF to the cut sciatic nerve using the same protocol resulted in survival of $62 \pm 5.1\%$ (mean $\pm$ s.d., $n=5$) of motor neurons on the lesioned side compared with the contralateral, non-lesioned side, a value not significantly different from controls (data not shown).

Our results demonstrate that motor neurons in newborn rats can transport BDNF and that BDNF has a significant survival-promoting effect in an axotomy-induced cell death paradigm. These results naturally raise the question of whether BDNF regulates motor neuron survival during development. To subserve the traditional role of a target-derived factor, BDNF should be synthesized in muscle. Whether BDNF is synthesized in physiologically significant levels in muscle is still not clear¹⁵. It is worth pointing out, however, that BDNF synthesis in several other locations, such as Schwann cells in peripheral nerve, astrocytes in spinal cord, or neurons that provide afferent input to motor neurons, might influence motor neuron survival. In

this regard, it is important to note that Schwann cells *in vitro* express BDNF messenger RNA as well as BDNF-like activity¹⁶ and a subset of dorsal root ganglion cells express high levels of BDNF mRNA¹⁷. Finally, BDNF might act indirectly, for example by stimulating Schwann cells to produce a motor neuron growth factor. Thus BDNF could work in a number of ways in addition to the traditional target-derived mechanism.

Do other recently identified neurotrophins promote motor neuron survival? In addition to BDNF, motor neurons are known to transport other members of the neurotrophin family including NT-3 (refs. 10, 11, 18, 19). Furthermore, motor neurons transiently express the low-affinity NGF receptor²⁰, P75, which binds all known neurotrophins^{7,21,22}. The patterns of expression of the trk family of tyrosine kinase receptors that may mediate the biological effects of neurotrophins are currently being investigated. Trk B, the putative BDNF receptor²²⁻²⁴ and trk C, the putative NT-3 receptor²⁵ are expressed by many cell types in the central nervous system, including motor neurons (S. Carroll, J. Milbrandt and W.D.S., unpublished data; and refs 25-27). But whether the forms of trk B and trk C expressed by motor neurons possess the tyrosine kinase domains necessary for biological action has not yet been determined. It is also important to note that there may be significant cross-reactivity among neurotrophins and trk receptor subtypes. Thus NT-3 activates trk²⁸, trk B^{22,24} and trk C²⁵, and NT-4/5 activates trk⁸ and trk B²⁹ *in vitro*. The ability of motor neurons to transport

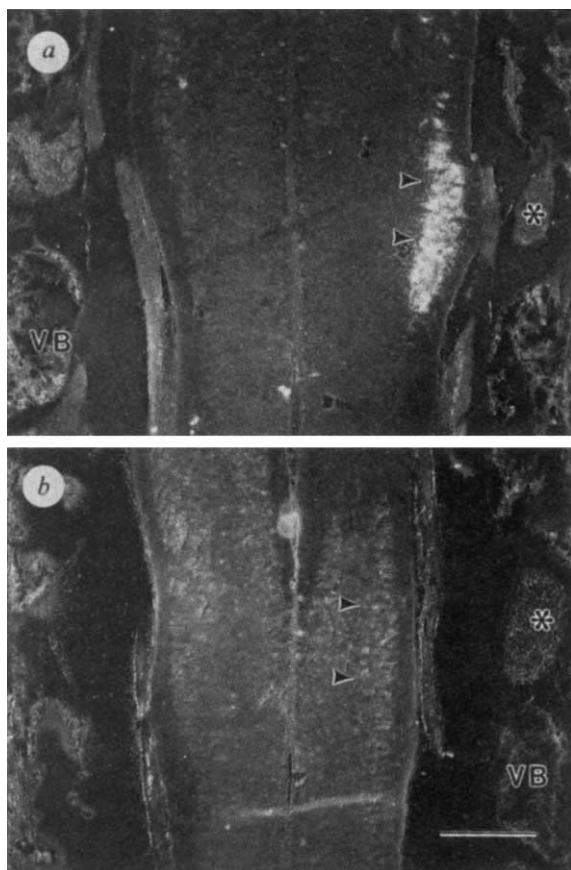


FIG. 1 Dark-field views of autoradiographs of lumbar spinal cords labelled after injection of ^{125}I -labelled BDNF into the hindleg muscles. Dense accumulation of silver grains was apparent over motor neurons in the ipsilateral (right) spinal cords of animals injected with ^{125}I -labelled BDNF alone (a), but no transport was observed in animals injected with a 50-fold excess of unlabelled BDNF (b). Arrowheads point to the motor columns in the L4-5 spinal cords and stars indicate the L2 dorsal root ganglia. Vertebrae (VB) tend to deflect light in dark-field illumination, resulting in some artefact. The scale bar in b represents 500 μm .

METHODS. The retrograde transport of BDNF by spinal motor neurons was examined by emulsion autoradiography as described¹⁰. BDNF was iodinated with Na^{125}I (Amersham) by the lactoperoxidase method¹². The iodinated protein was separated from free ^{125}I by G-25 Sephadex quick-spin columns (Boehringer Mannheim). A total of 2.2×10^6 c.p.m. (24.4 ng) of ^{125}I -labelled BDNF alone or with 50-fold excess of unlabelled BDNF were injected into the muscle of the right lower hindleg of 2-day-old rats through their foot pads ($n=4$). Animals were allowed to survive for 12 h after injection.

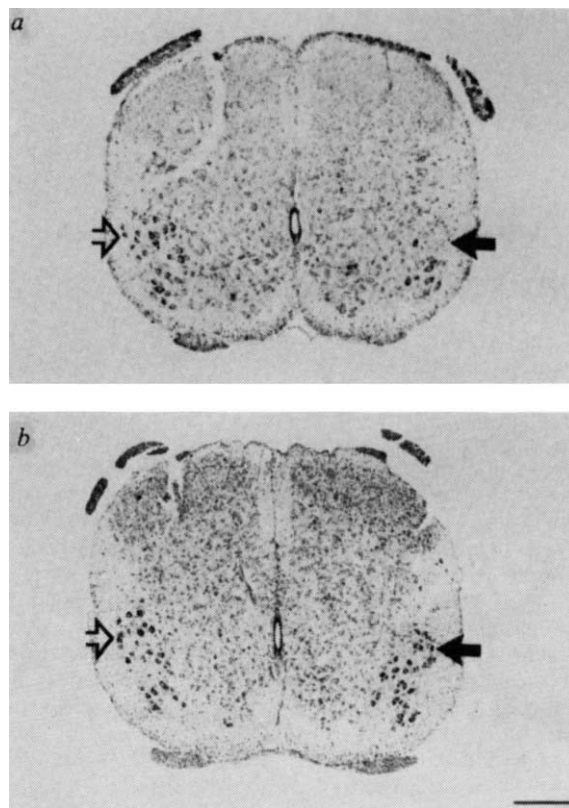


FIG. 2 Transverse sections of lumbar spinal cords from 6-day-old control (a) and BDNF-treated (b) rats after right sciatic nerve section on postnatal day 1. Open arrows indicate the non-lesioned side (left; marked by a slash in the dorsal horn) and solid arrows indicate the lesioned side (right). Approximately 40% of motor neurons were lost on the lesioned side in cytochrome c-treated rats. In contrast, only 8% of motor neurons were lost on the lesioned side in BDNF-treated rats. Scale bar, 200 μm .

METHODS. The L4-5 segments of spinal cord were removed, embedded in paraffin, sectioned at 8 μm and stained with thionin. Cells in the ventral horn with abundant cytoplasm identifiable as motor neurons were counted in every fifth section and the appropriate correction factors applied.

many neurotrophins that may crossreact with multiple receptors underscores the potential complexity of the neurotrophic dependency of this class of neurons.

In conclusion, we have shown that BDNF, a member of the neurotrophin family, has survival-promoting effects on motor neurons *in vivo*. This finding, together with those reported in the accompanying papers^{30,31} suggest that BDNF may be among the factors that regulate motor neuron survival during development. The identification of motor neuron growth factors is critical to clinical neurology because of the potential of such molecules to provide a treatment for human motor neuron diseases such as amyotrophic lateral sclerosis. □

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Brain-derived neurotrophic factor rescues developing avian motoneurons from cell death

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DURING normal vertebrate development, about half of spinal motoneurons are lost by a process of naturally occurring or programmed cell death^{1,2}. Additional developing motoneurons degenerate after the removal of targets^{3,4} or afferents⁵. Naturally occurring motoneuron death as well as motoneuron death after loss of targets or after axotomy can be prevented by *in vivo* treatment with putative target (muscle) derived or other neurotrophic agents⁶⁻⁸. Motoneurons can also be prevented from dying *in vitro*⁹⁻¹² and *in vivo* (Y.Q.-W., R.W., D.P., J. Johnson and L. Van Eldik, unpublished data and refs 7, 13, 14) by treatment with central nervous system extracts (brain or spinal cord) and purified central nervous system and glia-derived proteins. Here we report that *in vivo* treatment of chick embryos with brain-derived

neurotrophic factor rescues motoneurons from naturally occurring cell death. Furthermore, *in vivo* treatment with brain-derived neurotrophic factor (and nerve growth factor) also prevents the induced death of motoneurons that occurs following the removal of descending afferent input (deafferentation)⁵. These data indicate that members of the neurotrophin family can promote the survival of developing avian motoneurons.

Following the loss of about half of all postmitotic lumbosacral (LS) motoneurons between embryonic day (E)6 and E12 in the chick embryo, motoneuron numbers remain constant up to post-hatching stages^{1,6}. But by removing a few segments of the thoracic neural tube on E2 ('gap', Fig. 1), thereby preventing most spinal and supraspinal descending afferent inputs from later contacting motoneurons in the LS region, an additional 20-30% of the LS motoneurons present on E10 degenerate between E10 and E16 (Fig. 1; ref. 5). We have examined here the effects of the neurotrophins nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) on both of these types of motoneuron death in the chick embryo.

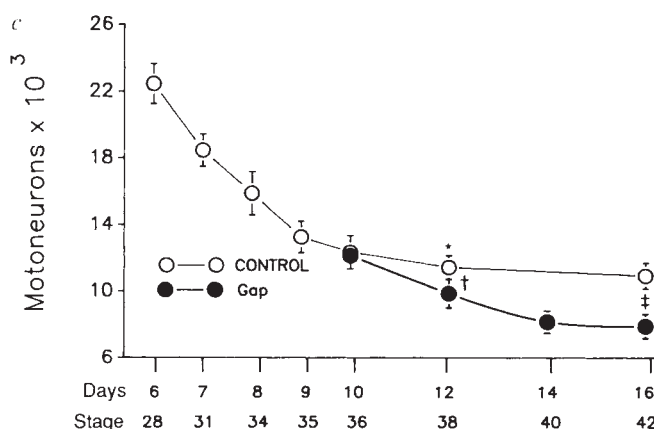
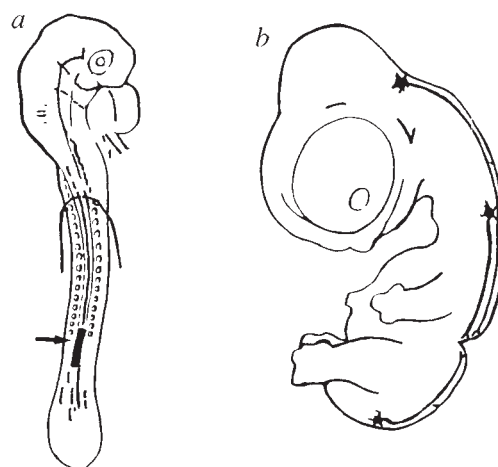


FIG. 1 Naturally occurring and deafferentation-induced death of LS motoneurons. *a*, A spinal 'gap' 2-4 somites long (arrow) was made in the presumptive thoracic neural tube on E2 as described⁵. *b*, As shown in this schematic illustration of an E5 embryo, this serves to eliminate descending supraspinal and propriospinal inputs to the LS spinal cord (deafferentation). *c*, Naturally occurring cell death begins on E6 and continues to about E12 during which time about half of the motoneurons degenerate. Deafferentation does not affect the death of motoneurons up to E10. But after deafferentation, there is a significant loss of motoneurons between E10 and E16 (ref. 5). After E16 the number of motoneurons remains constant at about 7,000-8,000. The number of embryos examined at each data point is 10-15. * $P < 0.01$ E9 versus E12 control; † $P < 0.01$; ‡ $P < 0.005$; control versus gap (*t*-tests). Motoneurons (mean $\pm$ s.d.) were counted as described^{4,28}.

Daily treatment of chick embryos *in ovo* with 5 μ g of BDNF from E6 to E9 resulted in a substantial reduction of naturally occurring motoneuron death (Table 1). An additional 15–16% of the motoneurons that would normally have died, survived (that is, BDNF rescued about one-third of the motoneurons that normally die between E6 and E10). In addition, as expected, two other populations of neurons responsive to BDNF *in vitro* and *in vivo*¹⁵ (dorsal root and nodose sensory ganglion cells, DRG and NDG), were also rescued from naturally occurring cell death in this situation (Table 1). Treatment with NGF rescued DRG neurons, but was without effect on the survival of motoneurons or neurons in the NDG. In addition to the effects of BDNF on survival, BDNF also influenced the growth (cell size) of DRG and NDG neurons, but not motoneurons (Table 1). By contrast, NGF treatment resulted in increased cell size of DRG neurons, but not of motoneurons or NDG neurons. We have not attempted to determine whether BDNF affects specific subpopulations of DRG neurons.

We have previously shown that deafferentation-induced motoneuron death⁵ can be prevented by *in vivo* treatment with CNS extracts and purified CNS- and glia-derived proteins (Y.Q.-W., R.W.O., D.P., J. Johnson and L. Van Eldik, unpublished data and refs 14, 15). For this reason, and because of our observation that BDNF can rescue motoneurons from normal cell death, we have also examined the effects of this brain-derived neurotrophin on motoneuron loss *in vivo* following deafferentation. Daily treatment of deafferented chick embryos with 10 μ g BDNF from E9 to E15 completely prevented the induced motoneuron loss that occurs in this situation (Fig. 2). Nerve growth factor, another member of the neurotrophin family, which has no effect on naturally occurring¹⁶ or axotomy-induced¹⁷ motoneuron death, was also effective in preventing deafferentation-induced motoneuron death (Fig. 2). In addition, BDNF, but not NGF, also prevented the small but statistically significant naturally occurring motoneuron death that occurs after E9 (Figs 1 and 2). Studies are in progress to examine the *in vivo* effects of two additional members of the neurotrophin family (NT-3

TABLE 1 Neuron numbers and cell size (nuclear diameter, μ m) (mean $\pm$ s.d.) on E10 following treatment of embryos with BDNF or NGF

	Control	BDNF	NGF
Number of moto-neurons	11,717 $\pm$ 1,103 (10)	15,221 $\pm$ 1,065* (20)	12,108 $\pm$ 1,103 (5)
Number of DRG	8,948 $\pm$ 894 (5)	11,602 $\pm$ 1,107† (10)	11,567 $\pm$ 720‡ (6)
Number of NDG	5,310 $\pm$ 383 (7)	6,734 $\pm$ 588§ (7)	4,873 $\pm$ 452 (6)
Motoneuron size	10.2 $\pm$ 1.1 (98)	10.3 $\pm$ 1.2 (100)	10.1 $\pm$ 1.3 (85)
DRG size	8.0 $\pm$ 1.3 (99)	8.6 $\pm$ 1.3 (100)	9.1 $\pm$ 1.5** (90)
NDG size	8.5 $\pm$ 1.1 (102)	9.0 $\pm$ 1.2¶ (99)	8.4 $\pm$ 1.0 (89)

Embryos were treated daily from E6 to E9 with NGF (5 μ g), BDNF (5 μ g) or cytochrome c (5 μ g) in 50 μ l of avian Tyrodes solution as described^{6,7}. Assuming a uniform distribution of trophic agent in the embryonic and extraembryonic compartments of the egg (total volume $\sim$ 60 ml), the amounts of trophic agent used here are in the range of 50 ng ml⁻¹. Purified murine β -NGF was supplied by E. Johnson and human recombinant BDNF by Amgen Inc. Embryos were killed on E10 and the LS spinal cord with attached DRG, and the ND ganglion were dissected, processed histologically and neurons counted as described^{4,28}. Motoneurons were counted in every 10th section through the entire LS region. Dorsal root ganglion neurons in LS segment number 3 and ND neurons were counted in every fifth section. Nuclear diameter was measured as described⁷. Numbers in parentheses are sample size (embryos or neurons). Neuron numbers in the NDG normally decrease by about 26% between E6 (7,400) and E10 (5,800). Following BDNF treatment, an additional 17% of the NDG cells that would have died, survived (that is BDNF rescued 68% of the cells that normally die). All statistical comparisons are between experimental and control groups. * $P < 0.001$, † $P < 0.001$, ‡ $P < 0.001$, § $P < 0.01$, || $P < 0.003$, ¶ $P < 0.01$, ** $P < 0.001$ (t-tests).

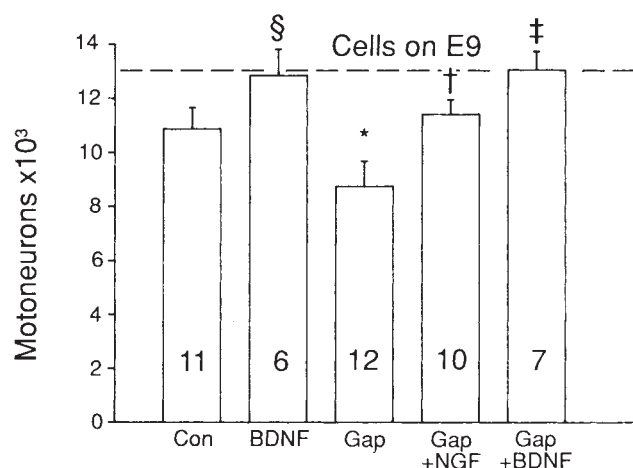


FIG. 2 Motoneuron numbers (mean $\pm$ s.d.) on E16 following treatment of deafferented (gap) and sham operated (control, con) embryos with BDNF or NGF. Embryos were treated daily with BDNF (10 μ g), NGF (10 μ g) or cytochrome c (10 μ g, control and gap) in 50 μ l of avian Tyrodes solution from E9 to E16 as described^{6,7}. The embryos in the Con and BDNF groups indicated here were unoperated (no gap). Assuming a uniform distribution of trophic agent in the embryonic and extraembryonic compartments of the egg (total volume, $\sim$ 60 ml), the amounts of trophic agent used here are in the range of 100 ng ml⁻¹. Embryos were killed on E16 and the LS spinal cord processed histologically and motoneurons counted as described^{4,28}. Numbers in the bars are sample sizes (number of embryos). The dashed line indicates the number of motoneurons present on E9 when treatment was begun. * $P < 0.005$ control versus gap; † $P < 0.003$ gap versus gap + NGF; ‡ $P < 0.001$ gap versus gap + BDNF; § $P < 0.01$ BDNF versus control.

and NT-4/5) on both naturally occurring and deafferentation-induced motoneuron death in the chick embryo.

The observation that members of the neurotrophin family can promote motoneuron survival *in vivo* was somewhat unexpected in that NGF, BDNF and NT-3 have all been reported to be ineffective in promoting motoneuron survival *in vitro*^{10–12}. In contrast, spinal motoneurons are known to transport NGF, BDNF and NT-3^{17–19} retrogradely and to express both low- and high-affinity NGF receptors²⁰, and motoneurons respond to NGF *in vitro* by enhanced neurite growth²¹. In addition, developing rat motoneurons express high levels of NT-3 messenger RNA²² and NGF, NT-3 and BDNF mRNAs are expressed in skeletal muscle^{23,24}. Accordingly, despite the negative *in vitro* data, it has been suspected for some time that neurotrophins may nonetheless play some part in motoneuron development or survival. The lack of effect of the neurotrophins on motoneuron survival *in vitro* is, on the face of it, difficult to reconcile with the present *in vivo* observations. One possibility is that the *in vivo* results reflect an indirect action of BDNF on motoneurons. We also cannot completely exclude the possibility that the *in vivo* effects reflect a nonphysiological, pharmacological action of neurotrophins on motoneurons. But we favour the idea that the lack of an *in vitro* effect may reflect the absence of cofactors or other conditions normally present *in vivo*, but absent *in vitro*, which are necessary for neurotrophins to act on motoneurons¹⁰. If this is correct, then it underscores the need to be exceedingly cautious in using *in vitro* assays alone for determining the range of cell types that may be responsive *in vivo* to suspected neurotrophic agents⁷.

In addition to the potential significance of these findings for understanding the regulation of normal motoneuron development and survival, these data, together with the related reports of Sendtner *et al.*²⁵ and Yan *et al.*²⁶, may also be potentially valuable in devising therapeutic strategies for treating moto-

neuron diseases and other pathological losses of motoneurons following injury²⁷. □

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Brain-derived neurotrophic factor prevents the death of motoneurons in newborn rats after nerve section

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MOTONEURONS innervating the skeletal musculature were among the first neurons shown to require the presence of their target cells to develop appropriately^{1,2}. But the characterization of molecules allowing motoneuron survival has been difficult. Ciliary neurotrophic factor prevents the death of motoneurons^{3–6}, but its gene is not expressed during development⁷. Although the presence of a neurotrophin receptor on developing motoneurons^{8–10} has suggested a role for neurotrophins, none could be shown to promote motoneuron survival *in vitro*³. We report here that brain-derived neurotrophic factor can prevent the death of axotomized motoneurons in newborn rats, suggesting a role for this neurotrophin for motoneuron survival *in vivo*.

The facial nerve of newborn rats was sectioned unilaterally at birth and the effects of three neurotrophins (nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3)) assessed 7 days later. This nerve was chosen for lesion and local application of neurotrophins as it contains only motor nerve fibres at the lesion site. Axons of proprioceptive sensory neurons (putative *in vivo* targets of BDNF and NT-3 (ref. 11)) were not lesioned (they are anatomically separated from this nerve) so that indirect effects through these cells are unlikely. Motoneurons that constitute the facial nucleus were counted on both sides. More than 80% of the axotomized motoneurons are lost when axotomy is done at birth^{5,12,13}. NGF, which has been shown to be ineffective in

TABLE 1 Motoneuron survival after lesion of the facial nerve in newborn rat: effects of neurotrophins

Treatment	Number of surviving neurons $\pm$ s.e.m.	
	Lesioned side	Control side
BSA ($n=8$)	844 $\pm$ 115	4,597 $\pm$ 222
NGF ($n=7$)	407 $\pm$ 127**	4,294 $\pm$ 227
BDNF ($n=9$)	2,222 $\pm$ 446*	4,534 $\pm$ 129
NT-3 ($n=10$)	1,221 $\pm$ 71*	4,510 $\pm$ 271

Transsection of the facial nerve was done as described previously⁵. Newborn Wistar rats were anaesthetized by hypothermia, the right facial nerve exposed at the foramen stylomastoideum and transected about 1 mm distal from this position. Gel foam (Spongostan, gift of K. Unsicker, Marburg, Germany) soaked in 30 μ l of PBS containing either 5 μ g of BSA (Cohn Fraction V, Sigma) or trophic factor was inserted at the site of lesion. Nerve growth factor (2.5 S) was isolated from submaxillary glands of adult male mice, as described²². Both recombinant mouse BDNF and NT-3 were produced in rabbit kidney cells infected with recombinant vaccinia viruses and purified as described²³. The skin was sutured by silk (Ethikon 3-0) and the animals returned to their mothers. On postnatal day 7 the animals were killed by ether overdose and perfused transcardially with 50 ml 4% formaldehyde. Brainstems were dissected, postfixed for 1 hour, rinsed with water, and dehydrated with increasing concentrations of ethanol (70–100%). After embedding with paraffin, serial sections (7 μ m) were made from the whole brain stem with a serial section microtome (Reichert-Jung 2050 supercut). The sections were stained with cresyl violet (Sigma), and facial motoneurons with clearly identifiable nuclei and nucleoli were counted on both sides at a magnification $\times 125$ in every fifth section as previously described⁵. The counts were not corrected for split nucleoli.

Different from BSA-treated animals at $P < 0.02^*$ or $P < 0.05^{**}$ (Student's *t*-test, 2-tailed).

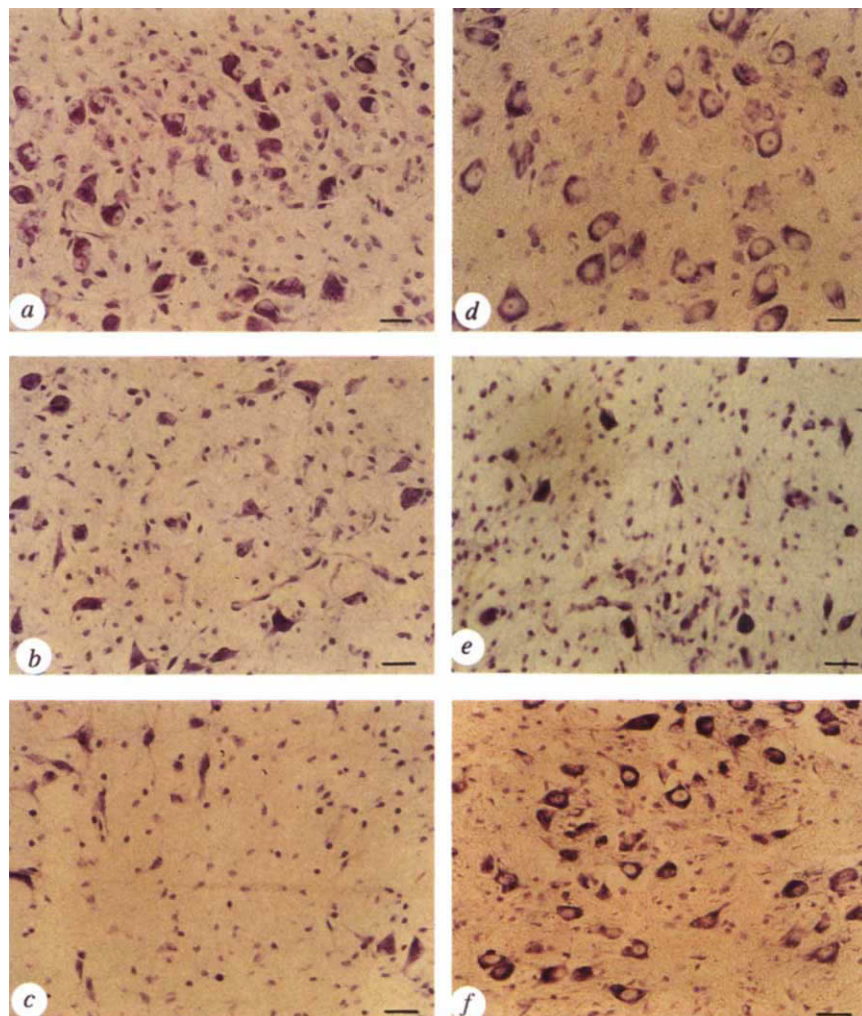
supporting motoneuron survival *in vivo*⁹, was used as a control, in addition to BSA. The application of NGF to the cut end of the facial nerve did not prevent the loss of motoneurons. In fact, fewer motoneurons were observed on the NGF-treated side as compared to BSA-treated animals ($P < 0.05$, Table 1).

In contrast to the animals treated with NGF, significant survival of axotomized motoneurons was observed when BDNF was applied to the transected facial nerve. On average, about 50% of the lesioned motoneurons were still alive 1 week after lesion (Table 1) which represents a surplus of 40% in comparison to NGF or 31% to BSA.

The survival of motoneurons was also enhanced following NT-3 treatment ($P < 0.02$ versus BSA-treated controls), though the effects were substantially smaller than those observed with BDNF (Table 1). In both BDNF- and NT-3-treated animals, the lesioned motoneuron cell bodies were smaller compared to unlesioned controls and showed typical reactive changes (Fig. 1). Although the lesioned motoneurons still remained clearly identifiable (see Fig. 1), the nuclei were displaced and chromatolysis was pronounced both in BDNF- and in NT-3-treated animals. But compared to control animals treated with BSA-gel foam, the nuclei appeared larger (Fig. 1).

These data show that molecules of the neurotrophin gene family can support motoneuron survival *in vivo*. In previous studies using purified motoneurons isolated from the chick spinal cord at 6 days of embryonic age, no survival effects could be observed with either BDNF or NT-3. This was not due to inadequate culture conditions, because the combination of ciliary neurotrophic factor (CNTF) and basic fibroblast growth factor (FGF) added to parallel cultures could rescue essentially all neurons³. Among the various explanations for the discrepancy with the present results are the possibility that some crucial cofactor, absent in culture, is required for the response of the neurotrophins to be seen. This cofactor might be another growth factor present *in vivo* or might be related to the presynaptic input of the motoneurons in the spinal cord. Alternatively, it is possible that motoneurons isolated from the chick embryo are not yet responsive to the neurotrophins or that they lose

FIG. 1 Morphology of facial motoneurons in the brainstem of 7-day-old rats after unilateral facial nerve section at birth. *a*, Lesioned side after BDNF treatment. *b*, Lesioned side after NT-3 treatment. *c*, Lesioned side after NGF treatment. *d*, Unlesioned contralateral side of the same animal as in *a*. *e*, Lesioned side after BSA treatment. *f*, Lesioned side after CNTF (5 μ g) treatment as a positive control. Scale bar, 50 μ m.



their responsiveness as a consequence of the isolation procedure. In any event, an important difference exists between motoneurons and sensory neurons cultured in isolation, in that all neurotrophins known so far support (to various degrees) the survival of neurons isolated from dorsal root ganglia.

In contrast to the CNTF gene⁷, both the NT-3 and BDNF genes are expressed during embryonic development¹⁴⁻¹⁶. For example, NT-3 messenger RNA is expressed at relatively high levels in the rat spinal cord between embryonic days 13 and 16, its expression decreasing gradually to low levels at birth¹⁵. Also, *in situ* hybridization studies have revealed that the spinal motoneurons themselves express the NT-3 gene^{15,16} and that BDNF expression is seen in neurons of the dorsal root ganglia in embryonic mice¹⁶. In addition to the central nervous system, measurable levels of BDNF mRNA have been detected in skeletal muscle¹⁴, as well as a variety of tissues in developing chick embryos including the skeletal musculature (K.-H. Herzog and Y.-A.B., unpublished results).

In the context of our results, it is worth noting that in adult rats there is substantial transport of radiolabelled BDNF, and to a lesser extent of NT-3, by spinal motoneurons¹⁷. But retrograde transport of neurotrophins is not always a reliable indicator of a biological response. In particular, retrograde transport of labelled NGF can be demonstrated in newborn rats (unlike in adults), but NGF does not rescue axotomized motoneurons (see ref. 9 and this study).

Finally, the expression of the BDNF gene is dramatically upregulated in the sciatic nerve of the adult rat with a delay of at least 3 days after lesion, suggesting that BDNF might play a

role in motor axon regeneration¹⁸. In contrast, CNTF is constitutively expressed in high amounts in myelinating Schwann cells of peripheral nerves of the adult rat¹⁹. After lesion, significant quantities of CNTF protein seem to persist for at least 1 week in the lesioned nerve and could be available to regenerating neurons¹⁹. Thus, a plausible scenario after peripheral nerve lesion would be that motoneurons are supported first by CNTF released from injured Schwann cells and subsequently by BDNF, the latter supporting the regeneration of motor axons to the periphery.

Our findings together with those reported by Oppenheim *et al.*²⁰ and Yan *et al.*²¹ indicate that motoneurons are responsive to BDNF *in vivo*. The observation that BDNF can support the survival of lesioned facial motoneurons that would die otherwise in the absence of survival factors indicates that this neurotrophin could have important functions on motoneurons, and mediate the regeneration of motor axons to the periphery after neuronal lesion. □

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Regions involved in the opening of CIC-2 chloride channel by voltage and cell volume

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REGULATION of cell volume is essential for every cell and is accomplished by the regulated loss or gain of intracellular ions or other osmolytes^{1–4}. Regulatory volume decrease often involves the parallel activation of potassium and chloride channels^{5–10}. Overexpression of P-glycoprotein leads to volume-activated Cl[−] currents^{11,12} but its physiological importance for volume regulation is unclear¹³. CIC-2 is a ubiquitously expressed Cl[−] channel¹⁴ activatable by non-physiologically strong hyperpolarization. We now show that CIC-2 can be activated by extracellular hypotonicity, which suggests that it has a widespread role in volume regulation. Domains necessary for activation by both voltage and volume are localized to the amino terminus. Mutations in an 'essential' region lead to constitutively open channels unresponsive to medium tonicity, whereas deletions in a 'modulating' region produce partially opened channels responsive to both hypo- and hypertonicity. These domains can be transplanted to different regions of the protein without loss of function.

Superfusion of *Xenopus* oocytes expressing CIC-2 with hypotonic solution produced a large increase in Cl[−] conductance. At isotonicity, CIC-2 Cl[−] current activates very slowly on strong hyperpolarization (Fig. 1a). Currents stimulated by

hypotonicity activate much faster and still display inward rectification (Fig. 1b). Although CIC-2 is normally closed at voltages less negative than −80 mV, hypotonicity induces a sizeable current in the physiological voltage range. This activation is fully reversible (Fig. 1c). Extracellular hypertonicity did not further reduce CIC-2 currents (data not shown). Roughly 10 min were needed before the effects of changes in osmolarity became apparent. This argues against a direct effect, and is compatible with slow intracellular changes in these large cells (such as cell swelling). Activation by hypotonicity was unchanged in Ca²⁺-free, EGTA-buffered saline, and also in oocytes additionally injected with EGTA (data not shown). Furthermore, the calcium ionophore A23187, which activates endogenous oocyte Cl[−] channels, did not elicit currents in oocytes injected with CIC-2 that were any larger than in control oocytes (data not shown). This argues against a role of calcium in channel activation.

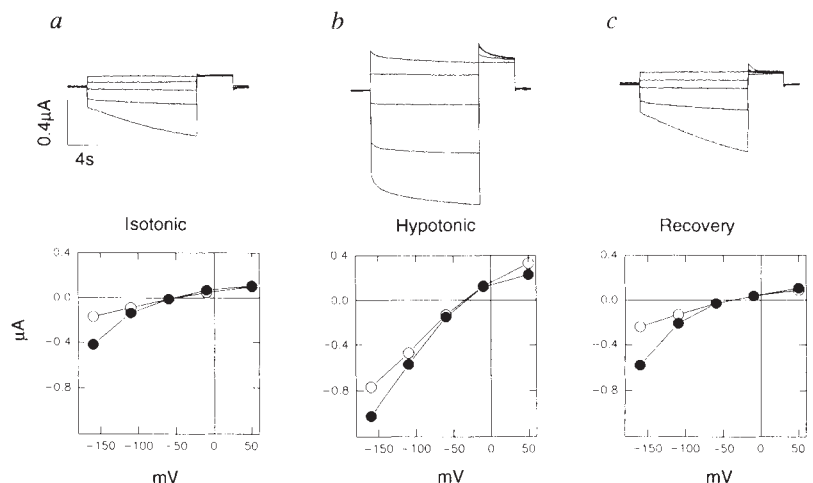
We constructed chimaeric proteins in which the region preceding the first transmembrane span D1 of CIC-2 was replaced by corresponding segments of the *Torpedo* electroplax channel CIC-0 (ref. 15) or the rat muscle channel CIC-1 (ref. 16). Expression of the CIC-(0/D1/2) construct (Fig. 2a) led to a large increase in membrane conductance, clamping the resting voltage to the Cl[−]-equilibrium potential. Similar but smaller currents were observed with the CIC-(1/D1/2) construct (Fig. 2b). With both chimaeras, the newly expressed conductance was almost time-independent and only slightly inward-rectifying, which contrasts with wild-type CIC-2. It fits, however, with the nearly linear instantaneous *I*–*V* relationship of CIC-2 observed after activation by hyperpolarization¹⁴. This suggests that a slow voltage-dependent 'gate' has been selectively removed by replacing the amino terminus.

Deletion of a large part (62 residues) of the amino terminus (mutant CIC-(0/10-W-63/2)) led to constitutively open channels, with currents similar to the CIC-(0/D1/2) chimaera. The extreme amino terminus of CIC-2, however, is not important for channel activation. Deletion of the first 15 amino acids, as well as several internal deletions between residues 6 and 20, resulted in wild-type activity.

Several internal deletions in a stretch of roughly 18 residues following Leu 21 led to an 'open' phenotype, defining a region that is essential for closing the channel at rest (Fig. 3a). Its amino-terminal border is remarkably sharp. Within this region, scattered deletions as small as four (or two) amino acids opened the channel. Neutralizing several charges either singly or in combination had no effect. But generating two new charges (mutant T26E/L27E) or increasing local hydrophobicity (mutant Q25L/T26L) led to the 'open' phenotype. Several 'open' mutants (listed in the legend to Fig. 3) were tested for their response to hyper- and hypotonicity: all were unresponsive (see Fig. 3b–d for mutant Δ(24–31)).

FIG. 1 Activation of CIC-2 by hypotonicity. Top, voltage-clamp traces; bottom, current–voltage relationships. ○, Currents present immediately after stepping to test voltage; ●, currents at the end of the 12-second test pulse. a, Currents under isotonic conditions (>10 min in $\frac{1}{2}$ ND96 + 100 mM sucrose); b, after hypotonic activation (19 min after removing sucrose), and c, after returning to isotonicity (15 min after adding back 100 mM sucrose).

METHODS. Capped mRNA was transcribed from CIC-2 clone ptm1-b12-2 (ref. 14) by T7 RNA polymerase after linearization with *Xba*I, about 5–10 ng of which were injected into *Xenopus* oocytes prepared and handled as described²⁷. They were stored and analysed in ND96 saline (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, pH 7.4). For analysis of hypotonic stress, they were first transferred to isotonic solution containing $\frac{1}{2}$ ND96 + 100 mM sucrose to eliminate potential effects of salt removal during the subsequent shift to $\frac{1}{2}$ ND96. Measurements were performed at room temperature (20–24 °C) using standard two-electrode voltage-clamp technique and pCLAMP software (Axon Instruments).



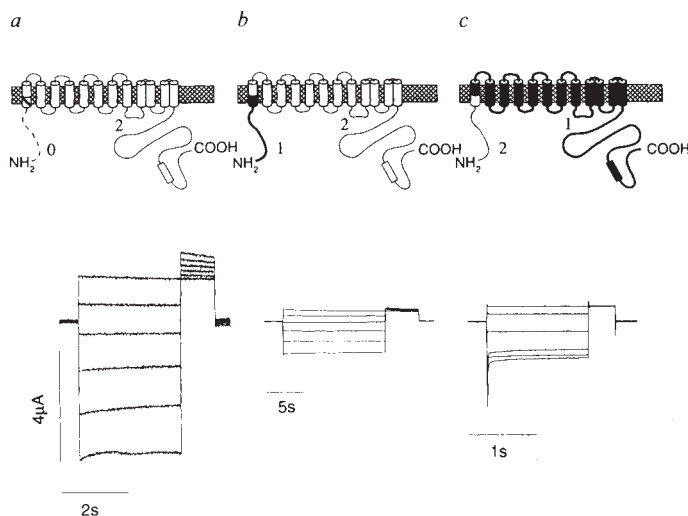
Immediately C-terminal to this 'essential' domain, we identified a region where deletions drastically changed channel activation without opening them constitutively (with the exception of $\Delta(40-43)$). These 'intermediate' mutants mediated significant Cl^- -current at resting voltages, showing fast activation (compared with wild-type) with hyperpolarization and steady-state inward-rectification (Fig. 3e). They could be further stimulated by hypotonic stress (Fig. 3f). In contrast to wild-type CIC-2 and 'open' mutants, they could be closed by hypertonicity up to the point of restoring wild-type CIC-2 characteristics (Fig. 3g). These 'intermediate' mutants resemble CIC-2 channels whose set points for cell volume activation are shifted towards hypertonicity (smaller cell volume), and so are partially activated at isotonicity. This suggests that the mechanism mediating volume

sensitivity can operate in both directions, and seems to exclude second messengers (such as calcium) whose concentrations can be raised but not lowered from resting minimal levels.

Structural analysis¹⁷ of the amino terminus predicts a helix-sheet-helix domain followed by a turn at about position 50-55, beyond which several mutations did not alter channel properties. The 'essential' region (where deletions produce the 'open' phenotype) is predicted to be a β -sheet whereas the 'modulating' region (where deletions result in 'intermediate' kinetics) lies within the second predicted helix. Both regions are highly conserved in the human CIC-2 protein (S.G., A.T. and T.J.J., unpublished results), the only difference being one conservative replacement (Glu 35 to Asp). As even the E35Q mutant had no effect, this is unlikely to have functional consequences.

FIG. 2 Electrophysiological properties of CIC chimaeric channels. Standard voltage-clamp experiments were done on chimaeric channels in which all amino acids preceding the (highly conserved) first transmembrane span D1 were exchanged between different channels. Schematic representations of the constructs are shown above the voltage-clamp traces. Putative transmembrane domains D1 through to D12 are shown as cylinders, and the conserved domain D13 is shown as a box. Recent transfer experiments described in the text suggest a cytoplasmic location of D13, according to model b of ref. 15. *a*, Construct CIC-(0/D1/2), in which the N terminus of CIC-0 precedes CIC-2; in contrast to CIC-2, the channel is constitutively open. *b*, CIC-(1/D1/2), with CIC-1 N terminus followed by CIC-2 backbone; this construct was expressed poorly and only in some experiments. *c*, CIC-(2/D1/1), N terminus of CIC-2 in front of CIC-1; the resulting current is similar to CIC-1 currents¹⁶, showing the typical deactivation at hyperpolarization and inward rectification in the positive voltage range. Voltage-clamp program: holding potential -30 mV; test pulses from $+40$ to -160 mV in 40 -mV steps, followed by a constant $+40$ mV pulse.

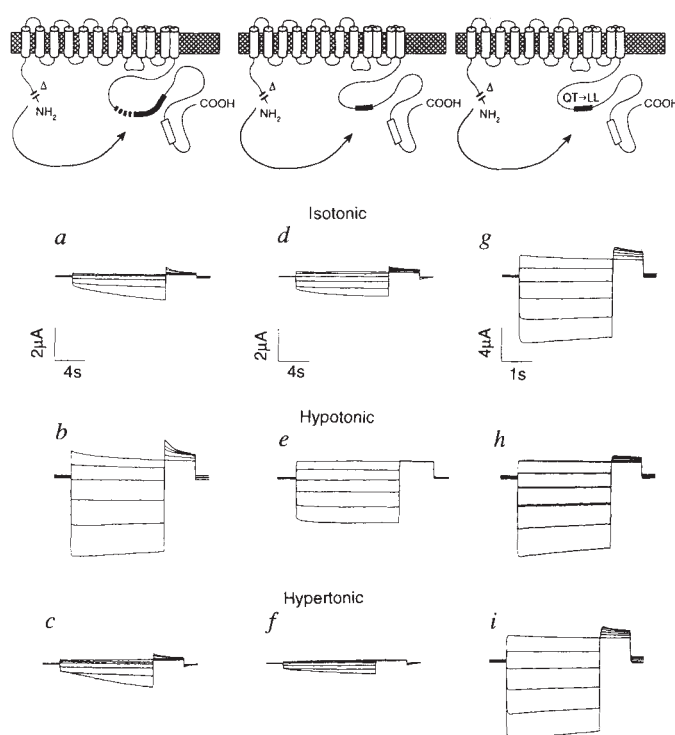
METHODS. Chimaeras were constructed by first introducing (using recombinant polymerase chain reaction²⁸ (PCR)) a silent *SpeI* restriction enzyme digestion site into all three channel cDNAs at a position coding for the conserved peptide sequence MALVS in D1. For CIC-0, Ala 60 was mutated in the same step to Leu to conform to D1 consensus sequence; Leu 56 in D1, which also differs from CIC-1 and CIC-2, was left unchanged. CIC-1 and CIC-2 N-terminal constructs always had CIC-0 5' non-coding sequence immediately preceding the initiator ATG which allows oocyte expression^{14,16}.



Chimaeras were constructed using this unique *SpeI* site. mRNA synthesis and analysis were as described in Fig. 1 legend. Experiments were done one day (*a* and *c*) or five days (*b*) after injection.

FIG. 4 Effects of transplantation of inactivating domains. Voltage-clamp experiments were done on *Xenopus* oocytes expressing mutant channels in which different parts of the N terminus were transferred into the cytoplasmic tail of an N-terminal deletion mutant: $\Delta(16-61)$ -XTB-CT (panels *a-c*), $\Delta(16-61)$ -SMB-CT (*d-f*), and $\Delta(16-61)$ -SMB-(QT $\rightarrow$ LL)-CT (*g-i*). Top, schematic representation of these mutants, which are described below. In the XTB mutant (left), an extended 'ball' (shown in black) containing a 'stuffer' (broken black region) has been inserted into the C-terminal Tail (CT); in the SMB mutant (centre), a Small 'Ball' has been transferred; in the SMB (QT $\rightarrow$ LL) mutant (right), this segment contains the Q25L/T26L mutation described in Fig. 3 legend. The open box represents the D13 domain. *a*, *d* and *g*, Isotonic conditions ($\frac{1}{2}$ ND96 + 120 mM sucrose; identical results were obtained with ND96); *b*, *e* and *h*, hypotonic conditions ($\frac{1}{2}$ ND96); *c*, *f* and *i*, hypertonic conditions (ND96 + 120 mM sucrose). In the XTB mutant, wild-type characteristics are fully restored; the SMB mutant has an 'intermediate' phenotype, and the SMB(QT $\rightarrow$ LL) mutant an 'open' phenotype.

METHODS. Transplantation mutants were constructed by ligating different PCR-amplified 5' sequences from CIC-2 (with added *NcoI* and *BstBI* sites at both ends) into the unique *NcoI* site of CIC-2 deletion mutant $\Delta(16-61)$. In the case of $\Delta(16-61)$ -SMB(QT $\rightarrow$ LL)-CT, the template for PCR was the mutant Q25L/T26L. For mutant $\Delta(16-61)$ -XTB-CT, this results in the insertion of GFEGTRPAARDRGPVALCTRRPGLRGQDGCGRGLSEPGAESRPEPEKQEEARGQCTE (Met 1-Arg 74) FEGH between His 695 and Gly 696; the 'stuffer' sequence preceding Met 1 was provided to increase potentially the mobility of the inserted sequence and stems mainly from the 5' normally untranslated region of CIC-2; for the SMB mutants, the insertion is GF(Glu 17-Lys 41)FEGH, with point mutations (already described) in the case of SMB(QT $\rightarrow$ LL). mRNA synthesis and measurements were done as for Fig. 1. The oocyte membrane potential was clamped to values between $+40$ and -160 mV. Oocytes were incubated for at least 15 min in the respective medium before starting measurements.



We asked whether the effect of deletions could be explained by a simple length effect. We inserted stretches of 33 or 66 amino acids into wild-type CIC-2 and into two deletion mutants after the 'modulating' region (Fig. 3a). There was no significant effect on voltage- or volume-dependent activation, and no consistent effects on the rate of deactivation (data not shown).

Next we took a constitutively 'open' deletion mutant and inserted different parts of the CIC-2 amino terminus between domains D12 and D13 (Fig. 4). We first inserted a large stretch fully encompassing the 'essential' and 'modulating' regions. This

fully restored both the voltage- and volume-dependence of channel activation (Fig. 4a-c). We then inserted a shorter stretch including the 'essential' region, but which lacked part of the 'modulating' region. This resulted in an 'intermediate' phenotype (Fig. 4d-f). Finally, an otherwise identical stretch from the 'open' mutant Q25L/T26L (Fig. 3) was unable to restore sensitivity to voltage and volume (Fig. 4g-i).

Thus the effects of amino-terminal determinants are largely position-independent. These experiments also support the postulated¹⁵ transmembrane topology of CIC Cl⁻ channels by

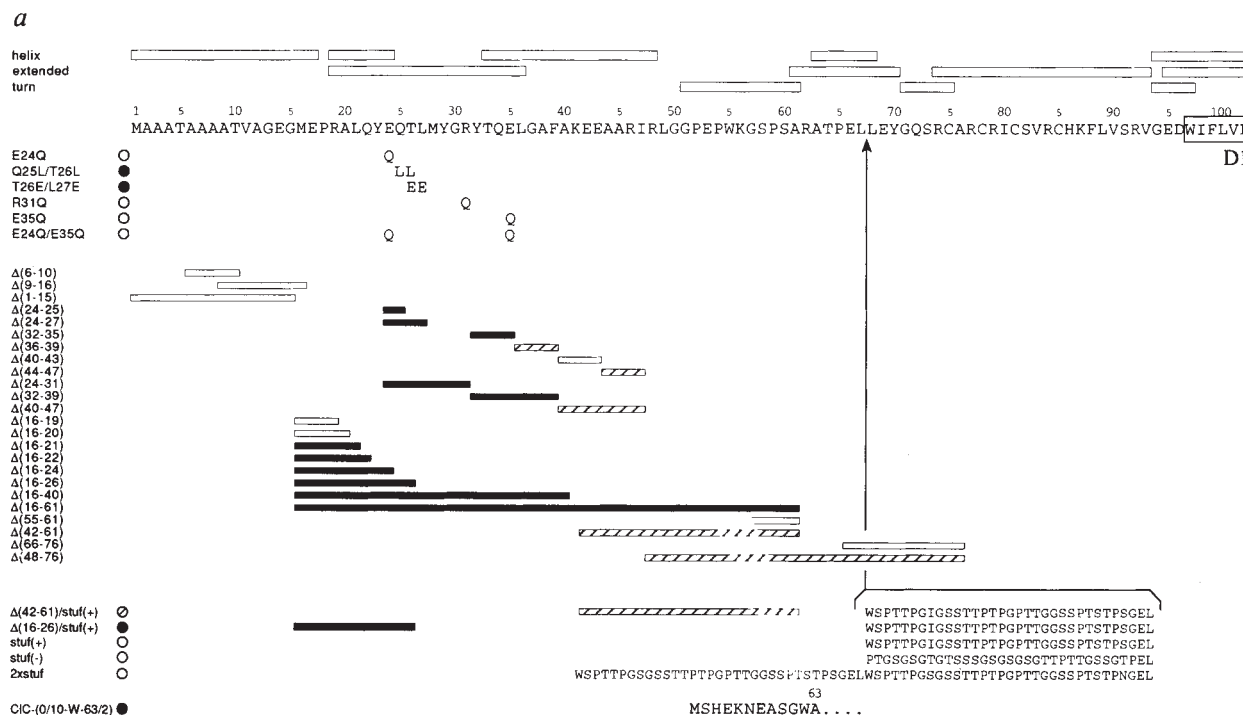


FIG. 3 Amino-terminal CIC-2 mutants and associated phenotypes. *a*, Schematic representation of mutants. At the top, structural predictions¹⁷ are shown for the N terminus of CIC-2, whose amino-acid sequence is given below. The start of the first transmembrane span D1 is boxed. Point mutations are indicated by the substituted amino acid, and deletion mutants by bars below the corresponding amino acids. These bars also indicate gross functional phenotype: blank, wild-type; black, 'open'; hatched, 'intermediate'. For other mutants, the phenotype is indicated by similar symbols (circles) after the construct name. The sequences for the stuffer fragments inserted after Leu 67 into wild-type CIC-2 and into two deletion mutants are shown with corresponding phenotype indicated by bars and/or circles. *b-g*, Representative voltage-clamp traces of N-terminal mutations. Typical measurements of oocytes injected with an 'open' mutant ($\Delta(24-31)$) (*b-d*) and an 'intermediate' mutant ($\Delta(44-47)$) (*e-g*) are shown. Experiments were done under isotonic conditions (*b, e*) ($\frac{1}{2}$ ND96 + 120 mM sucrose for >10 min); (*c, f*) hypotonic conditions ($\frac{1}{2}$ ND96 for 30 min (*c*) or 21 min (*f*)); (*d, g*) and hypertonic conditions (ND96 + 120 mM sucrose for 27 min (*d*) or 15 min (*g*)). Voltage was varied between +40 and -160 mV in 40 mV steps, followed by a constant step to +40 mV. Holding potential was equilibrium potential. The following 'open' mutants were tested for response to volume changes and were found to be unresponsive: $\Delta(16-21)$, $\Delta(16-22)$, $\Delta(16-61)$, $\Delta(24-31)$, $\Delta(32-35)$, Q25L/T26L, T26E/L27E.

METHODS. Internal deletion mutants and changes of single amino acids were constructed by recombinant PCR²⁸ using *Pfu* DNA polymerase (Stratagene). In $\Delta(1-15)$ the CIC-0 5' noncoding region was put (by PCR) immediately in front of the second ATG in frame (coding for Met 16). The chimaeric deletion mutant CIC-(O/10-W-63/2), in which the first ten amino acids of CIC-0 are fused to CIC-2 after amino acid 62, was constructed by ligation of the 5' *EcoRV* fragment of CIC-0 to the 3' *SmaI* fragment of CIC-2, which leads to an additional Trp residue at the ligation site. For 'stuffer' mutants, partially overlapping synthetic oligonucleotides (64 bases each) were annealed, extended by T7 DNA polymerase, self-ligated, cut with *SstI* (its recognition sequence was provided at both ends), and ligated into the 5' *SstI* site of CIC-2. Insertions of both orientations (*stuf*(+), *stuf*(-)) and a tandem head-to-tail insertion (2Xstuf) were obtained. Oligonucleotides

were designed to code for α -helix and β -sheet breaking, mostly polar amino acids on both strands. In the *stuf*(+) orientation, a *BstXI* site (with *SstI*-compatible overhang) is generated at the 5' end of the insertion, which was used to ligate its 3' fragment to the 5' *SstI* fragments of the corresponding deletion mutants for constructs $\Delta(16-26)$ *stuf*(+) and $\Delta(42-61)$ *stuf*(+). All constructs were confirmed by sequencing. Structural predictions¹⁷ were calculated using DNASTAR software (Madison, WI). Measurements were made as described for Fig. 1.

suggesting that the amino terminus and the region following D12 are on the same (cytoplasmic) side of the plasma membrane. The inactivating domain also functions when inserted after D13 (results not shown), effectively excluding D13 as a transmembrane domain.

To determine whether the CIC-2 amino terminus can impose slow activation by hyperpolarization upon another member of the CIC family, we put it in front of CIC-1 (construct CIC-(2/D1/1)). This did not significantly change its properties¹⁶ (Fig. 2c). Although it is not yet known whether hyperpolarization-induced slow activation^{15,18} of CIC-0 is caused by its amino terminus, we know from the properties of chimaera CIC-(0/D1/2) (Fig. 2a) that this domain cannot substitute for the CIC-2 amino terminus. Thus, the CIC-2 inactivating domain seems to interact specifically with a region (a putative 'receptor') present in CIC-2, but not in CIC-1.

Our findings are reminiscent of the 'ball-and-chain' model of inactivation¹⁹ experimentally verified^{20,21} for *Shaker* K⁺ channels. In this model, an amino-terminal 'ball' can block the open pore of the channel. It is tethered to the channel by a polypeptide 'chain'. The CIC-2 'ball' should correspond mainly to the 'essential' region, with the 'modulating' region possibly acting to modify binding affinity. In contrast to K⁺ channels, the 'ball' inactivates CIC-2 at rest and can be removed by voltage or hypotonicity (cell swelling).

With K⁺ channels, 'chain' length influences the rate of inactivation²⁰ by determining the space volume in which the 'ball' can move. Inactivation of CIC-2 is nearly two orders of magnitude slower than that of *Shaker B*, although their amino termini are comparable in length. Thus 'chain' length cannot be the common principle governing inactivation kinetics for both channels. Indeed, we observed no consistent effect with amino-terminal insertions, and inactivation times seemed unchanged in the transplantation mutants. Hence a different step (like a conformational change on binding the 'ball') seems to be rate-limiting.

The CIC-2 'ball' lacks known target sequences for regulatory mechanisms such as phosphorylation. Its small size seems to preclude simultaneous binding to the cytoskeleton and to the putative 'receptor'. Thus cell-swelling and voltage may act by changing the affinity of its 'receptor'.

Mutational analysis shows that the structural requirements on the CIC-2 'ball' are relaxed. This is akin to K⁺ channels, in which several mutations within the 'ball' have no effect²⁰, and for which the 'balls' from different channels share no homology²². Whereas very different K⁺ channels can be inactivated by the same 'ball'^{21–26}, the CIC-2 'ball' interacts quite specifically with a 'receptor' present only on CIC-2. Identification of this 'receptor', analysis of its interaction with the 'ball', and an understanding of the destabilisation of this interaction by changes in cell volume are important goals. □

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Phosphorylation of the *S. cerevisiae* Cdc25 in response to glucose results in its dissociation from Ras

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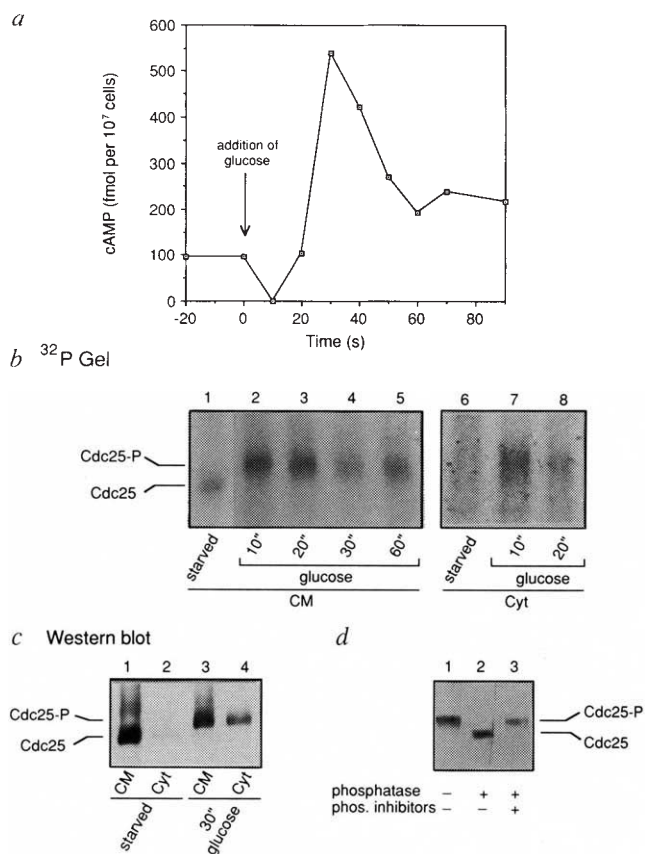
IN the yeast *Saccharomyces cerevisiae*, addition of glucose to starved cells triggers a transient rise in the intracellular level of cyclic AMP that induces a protein phosphorylation cascade¹. The glucose signal is processed by the Cdc25/Ras/adenylyl cyclase pathway², where the role of Cdc25 is to catalyse the GDP–GTP exchange on Ras³. The molecular mechanisms involved in the regulation of the activity of Cdc25 are unknown. We report here the use of highly selective anti-Cdc25 antibodies⁴ to demonstrate that Cdc25 is a phospho protein and that in response to glucose it is hyperphosphorylated, within seconds, by the cyclic AMP-dependent protein kinase. It is also demonstrated that, concomitantly with hyperphosphorylation, Cdc25 partially relocates to the cytoplasm, reducing its accessibility to membrane-bound Ras. These results are of general significance because of the highly conserved sequence of Ras–guanyl nucleotide exchange factors from yeasts to mammals.

Wild-type yeast cells (SP1)⁵ were labelled with ³²P-phosphoric acid before glucose starvation, and then incubated with glucose. Levels of cAMP and ³²P incorporation into immunoprecipitated Cdc25 were monitored in parallel. Figure 1 shows a temporal correlation between the diminished mobility of Cdc25 in the electrophoretic field, the extent of incorporation of ³²P into Cdc25 and the transient rise in intracellular cAMP levels. The incorporation of ³²P into Cdc25 peaks at 10 to 20 s (Fig. 1b, lanes 2, 3), and at the same time the cAMP level starts to rise sharply and peaks at 30 s (Fig. 1a). Thirty seconds after glucose addition, cAMP levels start to decrease and Cdc25 seems to be dephosphorylated in parallel to a lesser phosphorylated state (Fig. 1b, lane 4). In glucose-starved cells after 1 min the cAMP levels decay to an intermediate level, and, in parallel Cdc25 reaches an intermediate state of phosphorylation that is higher than the state of phosphorylation of Cdc25 (Fig. 1b, lanes 5 and 1, respectively). Exposure to glucose is also accompanied by a partial redistribution of the hyperphosphorylated Cdc25 to the cytoplasm whereas in the prestimulated state the less phosphorylated protein is exclusively localized to the membrane (Fig. 1b, lanes 1–3 and 6–8). This suggests that redistribution of Cdc25 to the cytoplasm results from its enhanced phosphorylation. The diminished electrophoretic mobility of the hyperphosphorylated Cdc25 generated in response to glucose and its redistribution to the cytoplasm is clearly observed in western blots using anti-Cdc25 antibodies (Fig. 1c). In these blots no change in the

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FIG. 1 Glucose-induced phosphorylation of Cdc25 and its relocalization to the cytoplasm in wild-type yeast cells (SP1). *a*, Glucose-induced cAMP signal after overnight starvation. *b*, Hyperphosphorylation of Cdc25 in response to glucose and its relocalization to the cytoplasm. Immunoprecipitates of ^{32}P -labelled crude membranes (CM; left panel) and of the cytoplasm (Cyt; right panel) before (starved) and after (10–60 s) stimulation with glucose. The hyperphosphorylated form of Cdc25 exhibits slower mobility and is denoted Cdc25-P; the less phosphorylated form is denoted Cdc25. *c*, Western blot analysis of phosphorylated Cdc25. An immunoblot of crude membranes (CM) and cytoplasm (Cyt) before (starved) and after (30 s) stimulation with glucose. *d*, Western blot analysis of phosphorylated Cdc25 treated with calf intestinal phosphatase. Membranes were either mock-treated (lane 1), treated with calf intestinal phosphatase (lane 2), or treated with phosphatase in the presence of phosphatase inhibitors (lane 3). Cdc25-P, the phosphorylated form; Cdc25, the dephosphorylated form.

METHODS. *a*, The cAMP assay was done as previously described¹. *b*, Cells grown overnight in a phosphate-free medium¹⁵ (1×10^7 cells per ml) were labelled with ^{32}P -phosphoric acid (0.5 mCi ml^{-1} ; Rotem industries, Dimona, Israel) for 120 min and then were starved of glucose for 18 h (0.02% glucose). After the cells were treated with 2% glucose for the indicated time intervals, lysates were prepared with glass beads as previously described⁴ with the addition of phosphatase inhibitors: 150 mM sodium vanadate, 50 mM sodium fluoride, 20 mM zinc chloride, 10 mM glycerol-2-phosphate, 30 mM *p*-nitrophenyl phosphate and 10 mM ammonium molybdate (all from Sigma). Lysates were centrifuged at $300,000g$ for 10 min at 4°C and the supernatant (cytoplasm; lanes Cyt) was separated from the pelleted material (crude membranes; lanes CM). Crude membranes were solubilized with 1% SDS and centrifuged at $12,000g$ for 5 min at room temperature. The supernatant was diluted 1:10 in HTN buffer (50 mM *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulphonic acid, pH 7.4, 1% Triton X-100 and 0.125 M NaCl containing protease and phosphatase inhibitors) and immunoprecipitated with anti-Cdc25 antibody as previously described⁴, except that immunoprecipitates were washed with HTN buffer containing 0.1% Triton. The cytoplasm was diluted 1:3 in HTN buffer and immunoprecipitated as described above. Precipitated proteins were subjected to SDS-PAGE (7.5% gel) and the gel was autoradiographed for 24 h. *c*, Cells were treated as described in *b* except for labelling with ^{32}P -phosphoric acid. Lysates were centrifuged at $300,000g$ for 10 min at 4°C . The pelleted material (crude membranes; lanes CM) and supernatant (cytoplasm; lanes Cyt) were separated, loaded on a 7.5% SDS-polyacrylamide gel and proteins were immunoblotted with anti-Cdc25 antibodies as previously described⁴. *d*, Glucose-induced SP1 membranes ($2.5 \mu\text{g}$ protein in $\sim 1 \mu\text{l}$) were treated with 2 mM EDTA (pH 12)⁴, diluted 1:10 in phosphatase buffer (100 mM Tris-HCl, at pH 9.6, 1% Triton, 2 mM MgCl_2 , 0.1 mM ZnCl_2) followed by the addition of 24 units of calf intestinal phosphatase (Boehringer Mannheim;



24 units μl^{-1}) to all but mock-treated samples. Samples containing phosphatase inhibitors were supplemented with a cocktail of phosphatase inhibitors (final concentrations, 5 mM sodium fluoride, 5 mM sodium phosphate, 10 mM sodium pyrophosphate, 10 mM ammonium molybdate, 5 mM EDTA, 5 mM EGTA). A cocktail of protease inhibitors⁴ was added to all samples. Mixtures were incubated for 1 h at 37°C , terminated with SDS-gel loading buffer, and boiled for 5 min before gel electrophoresis. This assay is a modification of an assay previously described¹⁶.

total amount of Cdc25 is observed within the duration of the glucose signalling.

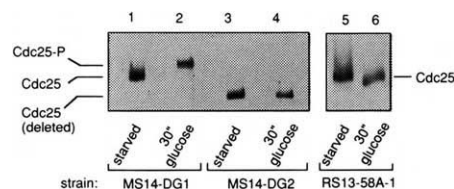
To confirm that the diminished electrophoretic mobility of Cdc25 is indeed a result of phosphorylation, glucose-stimulated SP1 membranes were treated with calf intestinal phosphatase (Fig. 1d). This treatment converted the slow-migrating form (lane 1) to the fast-migrating form (present during glucose starvation, lane 2). This conversion was completely blocked by phosphatase inhibitors (lane 3).

The transient nature of the cAMP signal is due to feedback inhibition by the cAMP-dependent protein kinase (cAPK)⁶ and Cdc25 contains six consensus sites for cAPK phosphorylation⁷. To determine whether cAPK directly phosphorylates Cdc25, we

constructed an in-frame deletion in the *CDC25* gene that eliminates four of the six consensus sites for cAPK phosphorylation. Figure 2 shows that in contrast to the glucose-induced mobility shift of the wild-type protein (lanes 1, 2), the deleted form of Cdc25 does not shift in the electrophoretic field as a result of glucose addition (lanes 3, 4). In this mutant strain the transient rise in cAMP is replaced by a constant lower level of cAMP sufficient to sustain normal growth, whereas the complete deletion of *CDC25* is lethal. The sustained level of cAMP is 150–180 fmol per 10^7 cells compared with 240–600 fmol cAMP per 10^7 cells in its isogenic wild-type strain and < 10 fmol cAMP per 10^7 cells in a strain in which the *CDC25* gene has been completely deleted. These results are in agreement with results

FIG. 2 Deletion of cAPK phosphorylation sites within Cdc25 and severely reduced cAPK activity nullify glucose-induced phosphorylation of Cdc25. An immunoblot of crude membranes before (starved) and after (30 s) stimulation with glucose. Lanes 1 and 2, MS14-DG1, strain MS14, disrupted in the *CDC25* gene¹⁷, carrying the YCplac33-*CDC25* plasmid (the complete *CDC25* gene). Lanes 3 and 4, MS14-DG2, strain MS14 carrying the YCplac33-*CDC25dH* plasmid (the deleted *CDC25* gene). Lanes 5 and 6, RS13-58A-1, a yeast mutant (isogenic to SP1) containing severely reduced but persistent activity of cAPK⁵; Cdc25-P, the hyperphosphorylated form; Cdc25, the less phosphorylated form; Cdc25 (deleted), the deleted form.

METHODS. Plasmid YCplac33-*CDC25* was constructed by cloning the full-length *CDC25* gene into the YCplac33 plasmid¹⁸. Plasmid YCplac33-*CDC25dH* was constructed by cloning a deleted *CDC25* gene (a *HpaI*-*HpaI* in-frame



deletion) into the YCplac33 plasmid. The resulting plasmid encodes a deleted protein (from amino acid 114 to amino acid 348 comprising 15% of the coding region).

previously described using the same deletion in the *CDC25* gene⁸. To determine further whether cAPK phosphorylates Cdc25, we have used a yeast mutant (isogenic to SP1) containing severely reduced but persistent activity of cAPK (RS13-58A-1)⁵. Addition of glucose to these cells does not induce a Cdc25 mobility shift (Fig. 2, lanes 5, 6) and these cells fail to show the descending portion of the typical glucose-induced cAMP signal as previously described⁶.

In membranes of SP1 cells when Ras proteins (Ras1 and Ras2) are immunoprecipitated with monoclonal anti-p21^{ras} antibodies (Y13-259), Cdc25 is co-immunoprecipitated in the unstimulated state but not in the glucose-stimulated state (Fig. 3, lanes 3, 4). Furthermore, when Cdc25 proteins are immunoprecipitated with anti-Cdc25 antibodies, the amount of Ras proteins co-immunoprecipitated in the glucose-stimulated state is well below that co-immunoprecipitated in the unstimulated state (Fig. 3, lanes 1, 2). These results indicate that reduced detection of Cdc25 or Ras in the immunoprecipitates reflects reduced association between the two proteins. Thus, glucose addition causes membrane-bound Cdc25 to relocate to the cytoplasm, reducing its accessibility to Ras proteins which are permanently bound to the membrane. It is also possible that enhanced phosphorylation of Cdc25 reduces its intrinsic affinity to Ras proteins, but this possibility can only be assessed by *in vitro* reconstitution.

Taken together, our results can be interpreted as follows (Fig. 4). The Cdc25 together with the glucose transporter unit are suggested to comprise the sensor unit for glucose, whereas the downstream element is the Ras-cyclase-cAPK. Initially, all the Cdc25 is membrane-associated and a significant portion of the Cdc25 is complexed to the GDP-bound Ras proteins⁹. On interaction of the complex with the glucose transporter unit, Cdc25 is stimulated to induce enhanced exchange of GDP by GTP on the complexed Ras protein, activating adenylyl cyclase (Cdc35/Cyr1). The cAMP produced activates cAPKs which in turn phosphorylate Cdc25, and as a result Cdc25 is partially released to the cytoplasm, therefore reducing the steady state level of the Cdc25-Ras (GDP) complex. Indeed the maximum phosphorylation of Cdc25 occurs at 10–20 s, yet cAMP levels are still basal (Fig. 1). One must however stress that the measurement of cAMP reflects the average of the whole cell volume whereas the phosphorylation event most probably senses the levels of cAMP at the compartment of the cyclase system. In hormone-sensitive systems of mammalian cells, the overall

FIG. 3 Co-immunoprecipitation of Ras proteins with Cdc25 is reduced after glucose signalling. An immunoblot of anti-Cdc25 and anti-Ras immunoprecipitates of crude membranes before (starved) and after (30 s) stimulation with glucose. Cdc25-P, the hyperphosphorylated form; Cdc25, the less phosphorylated form. The IgG heavy chain (anti-Cdc25 antibody and anti-Ras antibody; all lanes) is denoted by one asterisk; The light chain of IgG (only anti-Ras antibody; lanes 3, 4) is denoted by two asterisks. Positions of molecular mass markers (Amersham) are indicated on the right.

METHODS. Cells were treated as described in the legend to Fig. 1c. Crude membranes were treated with 2 mM EDTA (pH 12)⁴, diluted 1:10 in HTN buffer (1% Triton X-100) and immediately immunoprecipitated with anti-Cdc25 antibody (lanes 1 and 2) or anti-Ras antibody (lanes 3 and 4) according to the protocol described in the legend to Fig. 1b. Both types of immunoprecipitates were subjected to SDS-gel electrophoresis, blotted and separately probed with anti-Cdc25 antibody and with anti-Ras antibody. The top half of the figure represents the blot probed with anti-Cdc25 antibodies, whereas the bottom half represents the blot probed with anti-Ras antibodies. The two halves were pasted together to make the figure.

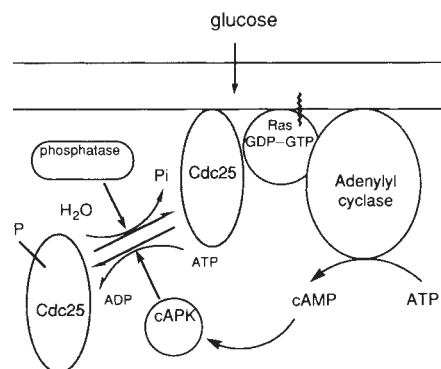
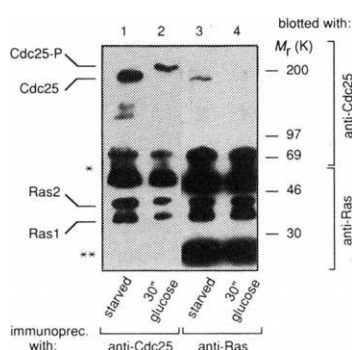


FIG. 4 Regulation of Cdc25 to Ras interaction by phosphorylation.

cAMP levels rise less and slower than at the compartment of the adenylyl cyclase¹⁰. Therefore it is possible that proteins proximal to adenylyl cyclase (for example cAPK) are activated within seconds after addition of glucose. On the basis of this assumption and of the results in Fig. 2, it is highly likely that Cdc25 is phosphorylated by cAPK before the intracellular average level of cAMP peaks. Thus initially one observes a burst of cAMP (Fig. 1) that reflects the high level of the Cdc25-Ras (GDP) complex, characteristic of the arrested (starved) state. Then, cAMP production levels off to an intermediate rate, reflecting the lower steady state level of the Cdc25-Ras (GDP) complex which results from the partial relocation of the hyperphosphorylated Cdc25 to the cytoplasm. This lowered steady-state level prevails as long as glucose is present (Fig. 1). When glucose is withdrawn, cAMP decreases to its basal level, Cdc25 is dephosphorylated to its less phosphorylated state and concomitantly relocates to the membrane ready for the next glucose signalling event. We have no information as to the intrinsic GDP-GTP exchange activity of the hyperphosphorylated Cdc25 compared to the less phosphorylated form of the protein. The resting (starved) form of Cdc25 is also phosphorylated and we have found no evidence for a totally unphosphorylated Cdc25. This observation, although preliminary, may suggest that limited phosphorylation at specific sites may be required for its interaction with Ras(GDP), whereas hyperphosphorylation reduces the interaction between the two proteins.

How are these results relevant to the recent findings that Cdc25 homologues exist in other yeasts¹¹, in *Drosophila*¹² and mammalian cells¹³ and that the carboxy-terminal domain, which is the catalytic domain, is highly conserved? In *Drosophila*, genetic evidence shows that the Cdc25 homologue (Sos) is downstream to a growth factor receptor tyrosine kinase (Sevenless)¹². The situation is probably similar in mammalian cells because stimulation of PC-12 cells by NGF leads to enhanced GDP-GTP exchange on p21^{ras} (ref. 14). These findings suggest that in these species, regulation of Cdc25 may also be mediated through phosphorylation. The Ras system in *S. cerevisiae* is highly similar to that of mammalian cells and *Drosophila* in its overall hierarchy and design but differs in the chemistry of the messengers. In *S. cerevisiae*, Ras proteins are responsible for proliferative signals as in mammalian cells but the effectors seem to differ. Similarly, both in yeasts (including *S. pombe* and *Candida albicans*) and in metazoans, Ras proteins are regulated by Cdc25 molecules which are conserved in the catalytic domains but differ in the regulatory amino-terminal domains which probably act as signal receivers. □

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Pit-1-dependent expression of the receptor for growth hormone releasing factor mediates pituitary cell growth

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IN Snell (dw) and Jackson (dw^J) dwarf mice, mutations in the gene encoding Pit-1, a tissue-specific POU-domain transcription factor, lead to the absence of somatotroph, lactotroph and thyrotroph cells^{1–6}. Pre-somatotroph proliferation is stimulated by increased intracellular levels of cyclic AMP, normally induced by growth hormone releasing factor (GRF; refs 7–17). Here we report the cloning of mouse and rat complementary DNAs encoding a new member of the seven-transmembrane-helix, G-protein-coupled receptor family restricted to the pituitary gland, which mediates increases in intracellular cAMP and cAMP-dependent gene transcription in response to GRF. The receptor is expressed in a spatial and temporal pattern corresponding precisely to growth hormone gene expression, and neither is expressed in dw/dw mice. The pituitary hypoplasia in these mice thus appears to be due, at least in part, to the absence of GRF receptor, which is in turn due to the absence of functional Pit-1.

Expression screening approaches have identified a new subfamily within the large family of G-protein-coupled seven-transmembrane-helix receptors (reviewed in ref. 18), including the secretin¹⁹, parathyroid²⁰, calcitonin²¹ and, most recently, vasoactive intestinal peptide (VIP) (ref. 22) receptors. As this subfamily may mediate trophic effects, we identified additional members from brain and anterior pituitary gland using degenerate oligonucleotide primers. A 410-base-pair (bp) fragment generated by polymerase chain reaction (PCR) from mouse anterior pituitary gland messenger RNA was used to screen cDNA libraries from rat and mouse anterior pituitary gland mRNA, and six cDNA clones were sequenced from each. The majority of mouse and rat cDNA isolates (Fig. 1a) encoded a 423-amino-acid product 35% identical to the VIP receptor in the predicted seven-transmembrane helix region, and least related to the calcitonin receptor (Fig. 1b). Alternative RNA processing produced an insertion at residue 325 (arrow in Fig. 1a) of the predicted rat receptor, which was particularly abundant in oestrogen-treated older animals. Additional alternative splicing products were detected in mouse (data not shown).

RNAse protection analysis showed that the gene was transcribed in the mouse pituitary gland, but not in the liver, lung, kidney, spleen, stomach, cerebral cortex, muscle, heart or testes (Fig. 2a), repeating the expression pattern of *pit-1* (refs 2, 3).

Northern blots showed transcripts of 2.1 and 2.0 kilobases (kb) in mouse pituitary RNA, but no transcripts in several permanent pituitary cell lines (GC, MMQ, 235) lacking GRF receptor activity (Fig. 2b). Whereas *pit-1* transcripts were first detected on e14.5 (embryonic day 14.5) (data not shown), transcripts encoding the cloned receptor first appeared on e16.5 in the central, caudal portion of the anterior pituitary gland (Fig. 2c).

To ascertain whether this protein was a GRF receptor, its coding sequence was placed in an expression vector driven by the cytomegalovirus promoter and transfected into COS, CV-1, HeLa and GC pituitary cells. As at the high assay background prevented unambiguous measurements of GRF binding (data not shown), the ability of transiently transfected receptors to produce GRF-inducible increases in intracellular cAMP levels was evaluated. In all cell lines tested, GRF caused a 16- to 30-fold elevation of intracellular cAMP (Fig. 3a), with half-maximal effects at $3\text{--}8 \times 10^{-10}$ M depending on the efficiency of transfection (Fig. 3b), and dose-response curves in accord with those seen in primary cultured pituitary cells (see for example ref. 14). In contrast, secretin, calcitonin and corticotropin-releasing hormone failed to increase intracellular cAMP levels. Because not all cells will express the receptor after transient transfection, the observed increase in cAMP levels underestimates the actual effects of GRF at a cellular level. To investigate signal transduction by the receptor further, cells were co-transfected with the receptor expression plasmid and a reporter controlled by a minimal prolactin promoter with well characterized cAMP response elements^{23–26}. Unlike the other peptides tested, GRF caused a 5- to 6-fold increase of reporter gene expression when added to these cells (Fig. 3c), with maximal stimulation at 10^{-7} to 10^{-8} M, comparable to the stimulation produced by forskolin (data not shown). These results indicate that the cloned receptor is the GRF receptor. All splice variants conferred GRF responsiveness by these assays (data not shown).

In both dwarf and wild-type mice, the developing pituitary gland contained comparable numbers of somatotroph precursor cells and expressed Pit-1 transcripts. In wild-type mice, *in situ* hybridization revealed that the growth hormone gene was first expressed in the anterior pituitary on e16, and was fully activated by e18.5 (Fig. 4a). In contrast, no growth hormone was detected in dw/dw animals, suggesting that Pit-1 is required, as predicted, for initial activation of the growth hormone gene^{2,27,28}. Wild-type mice also expressed the gene in the central caudal portion of the gland where Pit-1 and growth hormone were expressed (Fig. 4b). In dw/dw pituitary glands, however, GRF receptor transcripts could not be detected on e16–e18, although α -glycoprotein subunit transcripts were apparently equivalently expressed in dw/dw and wild-type pituitaries (Fig. 4c). Pit-1 thus directly or indirectly activates expression of the GRF receptor gene, which in turn is a key regulator of somatotroph cell proliferation.

The absence of the GRF receptor in the nascent somatotroph may, in part, explain the non-proliferation of these cells in the dw/dw mouse. Indeed, the GRF receptor gene is initially expressed at exactly the time GRF first appears in the fibres of the median eminence, from which GRF is delivered to the anterior pituitary gland via the portal vascular system²⁹, and the fetal pituitary gland first becomes responsive to GRF³⁰. A second genetic dwarf mouse (*lit/lit*), showing marked impairment of growth hormone and prolactin gene expression and a clearly hypoplastic anterior pituitary gland, may reflect a defect in the GRF receptor^{31,32}.

These data suggest that the failure to express the GRF receptor accounts, in part, for the proliferative defect in the Pit-1 defective Snell dwarf anterior pituitary gland, and provides a model for the coordinate control of cell proliferation and differentiated phenotype by tissue-specific transcription factors during mammalian organogenesis.

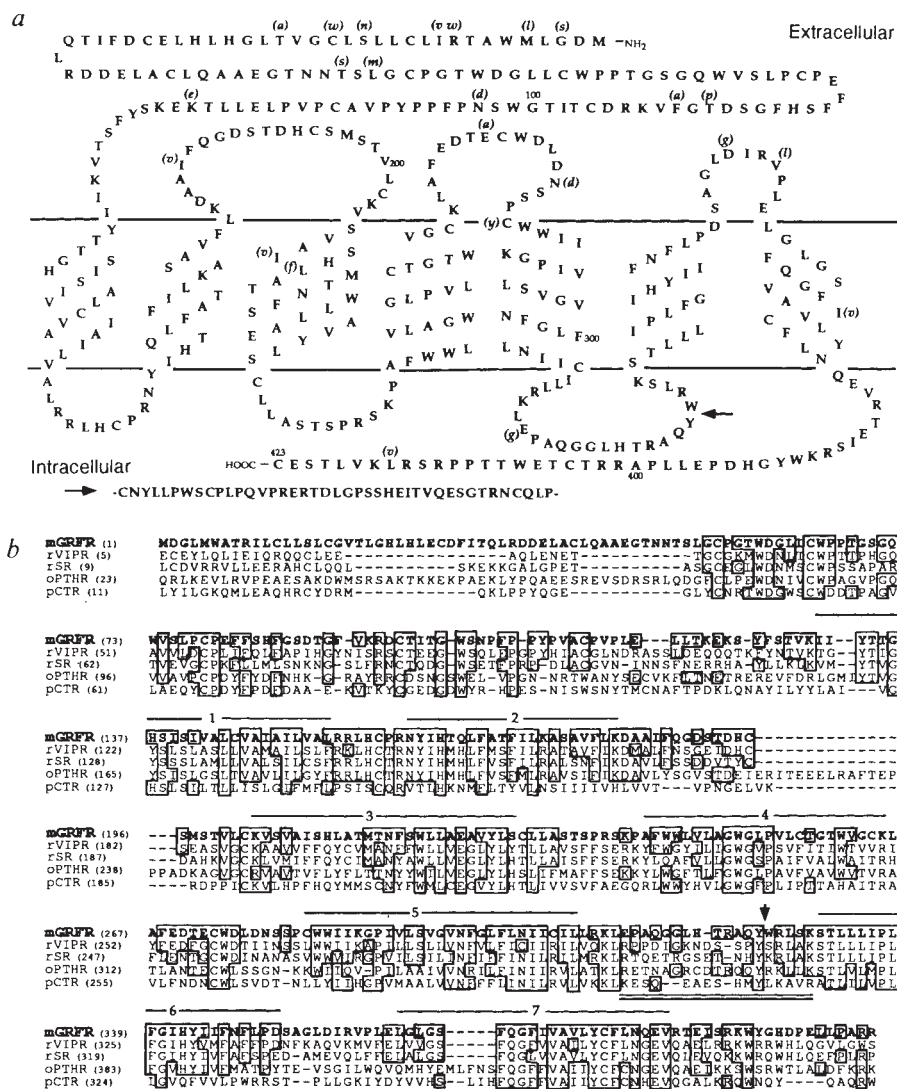
Note added in proof: Since submission of this letter, cloning of the rat and human GRF receptors has been reported³⁵. □

FIG. 1 Cloning of a novel receptor with a seven-transmembrane structure from anterior pituitary cDNA libraries. **a**, A 410-bp PCR fragment was generated from mouse pituitary cDNA using degenerate oligonucleotides corresponding to transmembrane regions 4 and 7 of three members of the G-protein-coupled transmembrane receptors. This sequence predicted an open reading frame with high homology to the corresponding region of the receptors for calcitonin, parathyroid hormone and secretin. Using this probe, six rat and six mouse cDNA clones were plaque-purified and the inserts sequenced. Three mouse pituitary cDNAs contained an open reading frame that encoded a 423-amino-acid protein with high homology to four known receptors containing seven putative transmembrane regions. Residues in parentheses (italics) are the only altered amino acids in rat cDNA. A schematic representation of the protein is shown; the precise boundaries of each putative transmembrane region are not known. Two rat cDNA clones contained a 41-amino-acid inset at residue 325, shown adjacent to the arrow. **b**, Alignment of the new receptor (mGRFR) with the rat (r) VIP, secretin (SR), parathyroid hormone (PTH), opossum (o), calcitonin (CT), porcine (p) receptor (-R) structures (RS); poor homology was noted in the N'- and C'-terminal portions of each protein (not shown). The putative G_s -coupling region is underlined, and transmembrane domains are indicated by solid bars.

METHODS. cDNA was generated using oligo(dT) and random hexamer primers and poly(A)⁺ RNA from mouse and rat anterior pituitary glands². Degenerate oligonucleotides (24 bases) corresponding to the conserved sequences in transmembrane regions 4 and 7 of the receptors for calcitonin, parathyroid hormone and secretin were used as PCR primers (C.P.C., unpublished). A 410-bp fragment was cloned, sequenced, and used as a probe to screen mouse λ gt11 and rat λ ZAP pituitary cDNA libraries (10⁶ plaques from each were screened). Twelve positive clones were purified and their inserts isolated and sequenced on both strands using the dideoxy nucleotide chain termination method³³.

FIG. 2 Expression patterns of cloned receptor mRNA. **a**, RNA blot analysis. Poly(A)⁺ RNA isolated from cultured cells and mouse pituitary gland was size-fractionated under denaturing conditions, and hybridized with a specific probe generated from a DNA fragment encompassing transmembrane regions 4 to 7. A discrete transcript doublet at 2.1 and 2.0 kb (4:1 ratio) was detected in size-fractionated RNA from anterior pituitary. No transcripts were detected in RNA from GC, 235 and MMQ pituitary cell lines. **b**, RNase protection analysis of receptor mRNA expression. Total RNA from various mouse tissues were used for RNase protection assays; a protected fragment of the expected size (400 nucleotides) was detected with RNA from pituitary, but not from any other tissue evaluated. **c**, *In situ* hybridization of cRNA probe to mouse e16.5 head, showing selective hybridization of the anterior pituitary (AP) gland.

METHODS. Total cellular and poly(A)⁺ selected RNA was prepared from various pituitary cell lines and mouse pituitary gland. RNA was size-fractionated under denaturing conditions, transferred to nitrocellulose filters, and hybridized to a 410-bp fragment labelled with [³²P]dCTP by random priming². RNase protection was done using a 450 nucleotide riboprobe complementary to the cloned 410-bp fragment and 10 μ g RNA from each tissue as described². *In situ* hybridization was done as before³ using a 410-bp cRNA probe; no signal was observed using sense strand cRNA.



Mouse GRFR and rat GRFR sequences are lodged with GenBank under accession numbers L07379 and L07380, respectively.

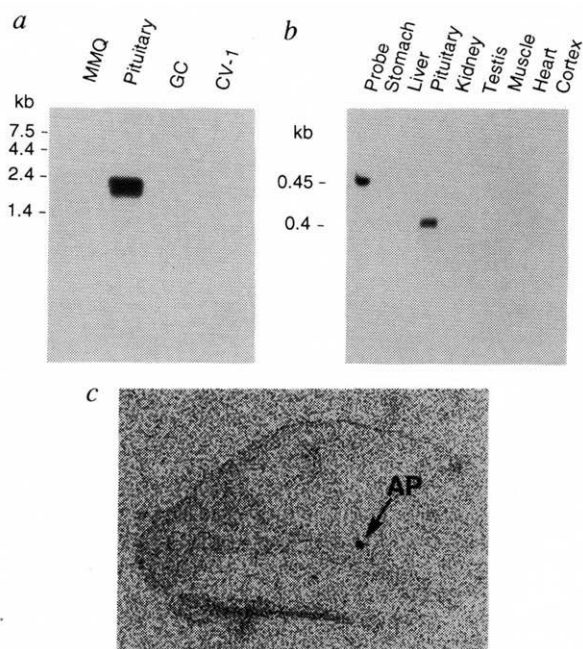
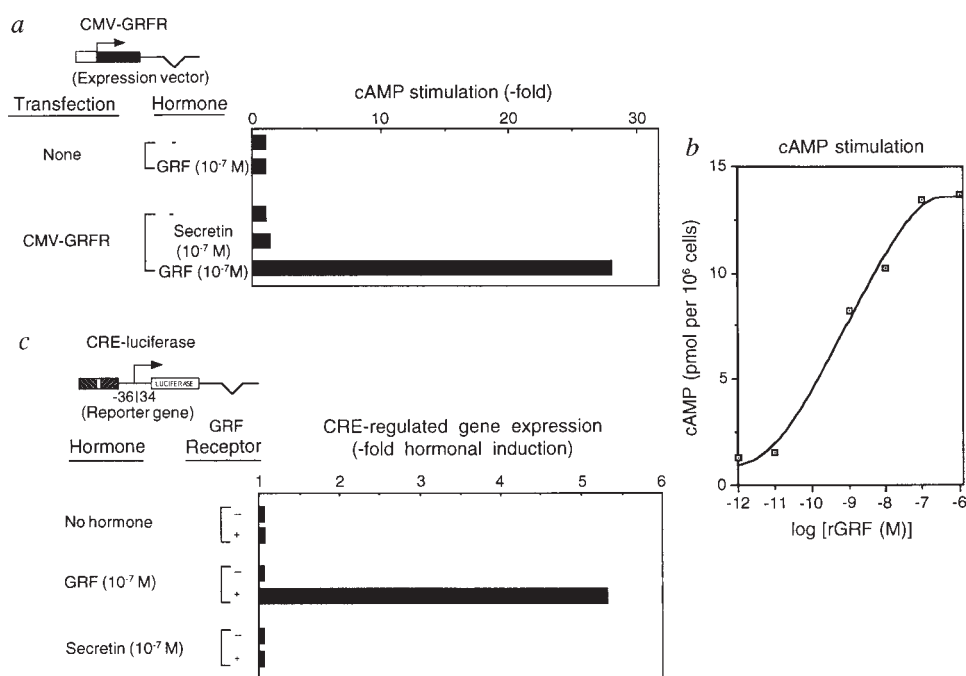


FIG. 3 Identification of the cloned growth hormone releasing hormone receptor. *a*, CV1 cells were transfected with a CMV-GRFR expression vector, or the vector alone, and effects of hormones on intracellular cAMP levels were determined (see Methods). Results are the average of duplicate determinations differing by <5%. cAMP levels were 0.5 pmol per 10^6 cells without GRF induction, increasing to 14.3 pmol in response to GRF. Stimulation was not observed in response to GRF in control transfected cells, or to 10^{-7} M corticotropin releasing factor (CRF) (not shown) and secretin in GRFR-expressing cells. Similar results were obtained in five experiments of similar design, using CV1 and COS7 cells as recipients. *b*, Dose response to GRF in CV1 cells transfected with the GRFR expression plasmid. Receptor-dependent stimulation of intracellular cAMP levels was determined in response to GRF, results on the average of duplicate determination differing by <5%. *c*, Activation of cAMP-responsive promoter elements in response to GRF.

The plasmid containing the CMV-GRF receptor transcription unit was cotransfected with plasmids containing a luciferase reporter gene under control of a minimal rat prolactin promoter (-36 to +34), with well characterized cAMP response elements (CRE)^{23,24}. Co-transfection with CMV vector containing no GRFR gene insert provided a control. GRH (10^{-7} , 10^{-9} M), or secretin (10^{-7} M), or forskolin (10^{-5} M; data not shown) were added to transfected cells 24 h before collection. Similar results were obtained in three independent experiments using GC, HeLa, and CV1 cells. The level of forskolin stimulation was actually slightly less than GRF-mediated stimulation in all experiments.

METHODS. *a* and *b*, Exponentially growing CV1 cells (60-mm dishes) were transfected with pCMV-GRFR expression plasmid DNA (5 μ g) using a calcium phosphate method². Dishes were rinsed with DMEM plus 10% FCS after



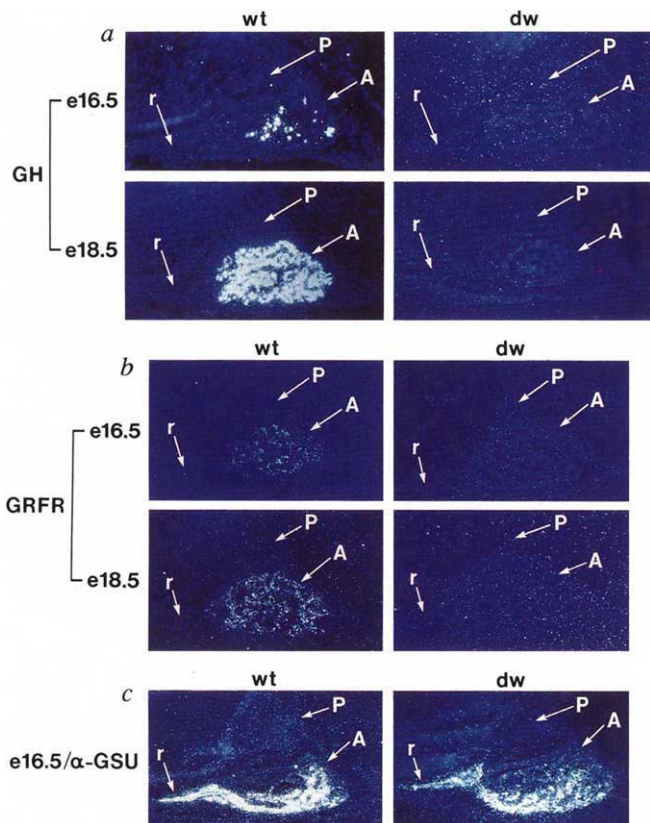
18 h. Two days later, plates were rinsed twice with DMEM/BSA (1 mg ml^{-1})/0.5 mM IBMX (3-isobutyl-1-methylxanthine). Cells were then incubated for 45 min at 37 °C in the same medium containing various peptides. Cells were lysed with 1 ml 95% ethanol, and cAMP in the supernatants was quantified using displacement of 8- ^3H -cAMP from a high-affinity binding protein as an assay (Amersham)³¹. Values are normalized. Experiments were done in CV1 and COS-7 cells. *c*, Transient co-transfection assays were performed as previously described². The CRE element used was: 5'-TTGGCTGACGTCAGAGAGAGAGGCCGCCCTTACGTCAGAGGCGAG-3', based on well-described response elements for CREB^{23,24}; 4.5 μ g CMV-GRFR and 1.5 μ g reporter plasmid were used for each transfection. Virtually identical results were obtained in GC, CV1 and HeLa lines. Cells were collected and luciferase units determined as before².

FIG. 4 Failure to activate GRF gene expression in the *dw/dw* mice. *a*, Ontogeny of Pit-1 and growth hormone gene activation in the *dw/dw* mouse. *In situ* hybridization was done using Pit-1 and growth hormone (GH) probes on serial sections of mouse anterior pituitary glands from wild-type (wt) and *dw/dw* mice (*dw*). By e16.5, growth hormone transcripts were detectable in a subset of cells in the *pit-1* positive area in wild-type animals, increasing by e18.5 to give maximal signals. In contrast, no GH transcripts could be detected in *dw/dw* mice on e16.5, e18.5 or at any other time in development. *Pit-1* reactivity was easily detected in *dw/dw* and wild-type glands in the central, caudal portion of the gland (not shown). *b*, Expression of GRFR transcripts was evaluated on each day between e15.5 and e18.5 in wild-type and *dw/dw* mouse pituitary glands. In the wild-type mouse, the region of GRF receptor hybridization precisely overlapped the region of Pit-1 and growth hormone hybridization, initially detected on e16.5. In *dw/dw* anterior pituitary glands, no GRF receptor signal was detected, even with long exposure either on one initial day of activation (e16.5) or on any subsequent day. *c*, α -Glycoprotein subunit (α -GSU) transcripts were detected to the same extent in wild-type and dwarf (*dw/dw*) mice, in rostral tip (r) and central caudal portions of anterior gland (a). P, posterior lobe.

METHODS. Embryos from wild-type or *dw/dw* mice in ice-cold PBS were transferred to 4% paraformaldehyde fixative solution and serially sectioned at 10 μ m as before³. Hybridizations were done using 5×10^6 c.p.m. ml^{-1} ^{35}S -labelled antisense (or sense) RNA probes of GH, Pit-1 or GRFR (410 nucleotides) as already described. After hybridization sections were washed and dehydrated and then exposed to X-ray film (Amersham). After autoradiography, slides were defatted, coated with emulsion (NTB-2), and developed after 10–14 d of exposure. No hybridization was seen with comparable levels of sense-strand RNA probe.

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ERRATA

Cloning and expression of a rat brain L-glutamate transporter

Gilia Pines, Niels C. Danbolt, Magnar Bjørås, Yumin Zhang, Annie Bendahan, Lars Eide, Hermann Koepsell, Jon Storm-Mathisen, Erling Seeberg & Baruch I. Kanner
Nature **360**, 464-467 (1992)

THE third author's name was shown incorrectly in this letter. It should read Magnar Bjørås, as above.

In addition, Fig. 2b is not, as stated, a western blot but is an autoradiogram of an SDS-polyacrylamide gel electrophoresis gel.

Addition and subtraction by human infants

Karen Wynn

Nature **358**, 749-750 (1992)

IN Table 1 of this letter, the looking times for experiment 2, test trials of group 2-1 are reversed. They should indicate that LT(1) = 8.05 and LT(2) = 10.98.

Importance of habitat saturation and territory quality for evolution of cooperative breeding in the Seychelles warbler

Jan Komdeur

Nature **358**, 493-495 (1992)

IN this letter dealing with cooperative breeding on the Cousin and Cousine Islands in the Seychelles, in four places in the text Cousin is referred to instead of Cousine. These are in the following places: Line 22 in the right-hand column on page 494; and in lines 1, 4 and 11 in the left-hand column on page 495.

Alterations in a yeast protein resembling HIV Tat-binding protein relieve requirement for an acidic activation domain in GAL4

Jonathan C. Swaffield, Jacqueline F. Bromberg & Stephen A. Johnston
Nature **357**, 698-700 (1992)

IN Fig. 2 of this letter, the last five amino acid residues (401-405) were not shown. They are "AKLFK".

CORRECTIONS

New 'phantom' dinoflagellate is the causative agent of major estuarine fish kills

JoAnn M. Burkholder, Edward J. Noga, Cecil H. Hobbs & Howard B. Glasgow Jr

Nature **358**, 407-410 (1992)

IN this letter in the 30 July issue, Stephen A. Smith (Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute, Blacksburg, Virginia 24061-0442, USA), a former collaborator of the second author, was inadvertently omitted and should be added as a fifth author. The Office of Sea Grant (NOAA, US Department of Commerce, and the UNC Sea Grant College), and the North Carolina Agricultural Research Foundation should be acknowledged for their contributions to funding support.

Rapid changes of mitochondrial Ca²⁺ revealed by specifically targeted recombinant aequorin

Rosario Rizzuto, Alec W. M. Simpson, Marisa Brini & Tullio Pozzan

Nature **358**, 325-327 (1992)

THE full address for correspondence (with R.R.) relating to this letter is: The Department of Biomedical Sciences, CNR Center for the Study of Mitochondrial Physiology, University of Padova, Via Trieste 75, 35121 Padova, Italy. In addition, the following sentence should be added to Acknowledgements: The financial support of the V AIDS project is also acknowledged.

A new type of synthetic peptide library for identifying ligand-binding activity

Kit S. Lam, Sydney E. Salmon, Evan M. Hersh, Victor J. Hruby, Wieslaw M. Kazmeierski & Richard J. Knapp

Nature **354**, 82-84 (1991)

IN this paper we inadvertently omitted to cite the work of Furka and colleagues (A. Furka, F. Sebestyen, M. Asgedom and G. Dibo *14th Int. Congr. Biochem.* FR013; 1988), who independently described a similar synthetic method for producing multiple peptide sequences (which we called "split synthesis"). However, Furka *et al.* did not describe the concept of 'one bead, one peptide' which was central to our approach.